

Cell cycle-dependent changes of the pericentriolar material

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Abbreviations

ACN	Acetonitrile
AKAP	A-kinase anchor protein
APC/C	Anaphase promoting complex/cyclosome
Asp	Abnormal spindle
ATP	Adenosine-5'-triphosphate
BCA	Bicinchoninic acid
BRCA1	Breast cancer suppressor protein 1
BRCA2	Breast cancer suppressor protein 2
BSA	Bovine serum albumine
CaMKII	Calmodulin dependent protein kinase II
CHO	Chinese hamster ovary
CPAP	Centrosomal P4.1-associated protein
Cdk	Cyclin dependent kinase
cDNA	Complementary DNA
C-Nap1	Centrosomal Nek2-associated protein 1
DAPI	4',6-diamidino-2-phenylindole
dH ₂ O	de-ionised water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide-5'-triphosphate
D-TACC	Drosophila-transforming acidic coiled-coil
DTT	1,4, dithiothreitol
EBNA-2	Epstein-Barr nuclear antigen 2
ECL	Enhanced chemiluminescence
<i>E. coli</i>	<i>Escherichia coli</i>
EM	Energy mix
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene glycol-bis-(β Aminoethyl ether)-N, N, N', N'-tetra acetic acid
FACS	Flow analysis cell sorting
FITC	Fluorescein isothiocyanate
GCP	γ -tubulin complex protein
GTP	Guanosine-5'-triphosphate
γ -TuRC	γ -tubulin ring complex
γ -TuSC	γ -tubulin small complex

Grips	γ -tubulin ring proteins
GST	Glutathione S transferase
kb	kilobase
kDa	Kilodalton
LB	Luria Bertani
LPC	Leupeptin, pepstatin, chymostatin
HCA66	Hepatocellular carcinoma antigen 66
HCG	Human chorionic gonadotropin
HeLa	Henrietta Lacks
HEPES	<i>N</i> -[2-Hydroxyethyl] piperazine- <i>N'</i> -[2-ethanesulfonic acid]
HRP	Horseradish peroxidase
Hsp	Heat shock protein
IL1	Intermediate line 1
IL2	Intermediate line 2
IMAGE	Integrated molecular analysis of genome and their expression
IP1	Inner plaque 1
IP2	Inner plaque 2
IPTG	Isopropyl β -D-thiogalactopyranoside
MALDI-tof	Matrix-assisted laser desorption/ionisation-time of flight
MCC1	Mutated in colorectal cancer 1
MB	Migration buffer
MOWSE	Molecular weight search
MTOC	Microtubule organising centre
Nek2	Nima related kinase 2
NPM/B23	Nucleophosmin/B23
NuMA	Nuclear and mitotic apparatus
OD	Optical density
Odf2	Outer dense fiber 2
PACT	pericentrin AKAP450 centrosome targeting
PARP-1	Poly (ADP ribosyl) polymerase 1
PBS	Phosphate-buffered saline
PBStw	PBS Tween
PCM	Pericentriolar material
PCR	Polymerase chain reaction
PKA	Protein kinase A
PKC ϵ	Protein kinase C ϵ
PKN	Protein kinase N

PMSF	Phenylmethanesulfonyl fluoride
PMSG	Pregnant mare serum gonadotropin
PIPES	Piperazine, N, N'-bis-[2-ethanesulfonic acid]
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcriptase PCR
SCF	Skp1-Cul1-F box
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Silencing RNA
SPB	Spindle pole body
TAE	Tris acetate EDTA
TCP-1	Tailless complex polypeptide-1
TEMED	<i>N,N,N',N'</i> - tetramethylethylenediamine
TPR	Tetra trico peptide repeat
Tris	Tris (hydroxymethyl) aminomethane
UTR	Untranslated region
U.V.	Ultraviolet
x4.1R ¹³⁵	<i>Xenopus laevis</i> 4.1R ¹³⁵

Table of content

ACKNOWLEDGMENTS	iii
ABBREVIATIONS	iv
TABLE OF CONTENT	vii
LIST OF FIGURES	xiii
LIST OF TABLES	xiv
ABSTRACT	xv
INTRODUCTION	1
I STRUCTURE OF CENTROSOMES AND SPINDLE POLE BODIES.....	1
A – CENTROSOME STRUCTURE	2
B – THE SPINDLE POLE BODY: AN OVERVIEW	6
1) <i>Ultrastructure of the spindle pole body of <i>Saccharomyces cerevisiae</i></i>	6
2) <i>Molecular composition of the SPB</i>	7
3) <i>Tub4p and the Tub4p complex</i>	9
4) <i>The role of the Spc110p protein</i>	10
5) <i>Spc72p anchors the Tub4p complex at the outer plaque?</i>	10
II BIOLOGICAL ROLES OF THE CENTROSOME	11
A - THE CENTROSOME AS A MICROTUBULE ORGANISING CENTRE.....	11
B - THE ROLE OF γ -TUBULIN AND THE γ -TUBULIN RING COMPLEX IN MICROTUBULE NUCLEATION	13
1) <i>The γ-tubulin ring complex</i>	14
2) <i>The γ-tubulin small complex</i>	15
3) <i>How is the γ-TuRC recruited to and anchored at the centrosome?</i>	17
C - ANCHORING MICROTUBULES TO THE CENTROSOME	18
D - THE ROLE OF THE CENTROSOME IN THE FORMATION AND POSITIONING OF THE MITOTIC SPINDLE.....	19
1) <i>The centrosome is dispensable for spindle assembly</i>	19

2) <i>The 4.1R protein is present at the centrosome</i>	21
E - ROLE OF THE CENTROSOME DURING CYTOKINESIS	24
F - THE CENTROSOME IS REQUIRED FOR THE G1/S TRANSITION	26
G - REGULATION OF CENTROSOME FUNCTIONS: ANCHORING OF REGULATORY MOLECULES, THE ROLE OF AKAPs	27
III CENTROSOME CYCLE	28
A - THE CENTROSOME DUPLICATION.....	30
B - THE PROTEINS REGULATING THE CENTROSOME CYCLE	32
1) <i>The kinases regulating the centrosome cycle</i>	34
a) The role of CDK2 in the centrosome cycle	34
NPM/B23 is a substrate of Cdk2	35
b) MPS1 is required for centrosome duplication.....	36
c) ZYG-1, a unique kinase involved in centriole duplication	37
d) The role of calcium and calmodulin-dependent kinases in the centrosome cycle.....	37
e) Polo and polo-like kinases.....	39
f) Aurora A regulates centrosome replication.....	39
2) <i>Ubiquitin-mediated proteolysis and regulation of the centrosome cycle</i>	40
a) Role of the SCF E3 ubiquitin ligase in the centrosome cycle.....	40
b) The APC/C is involved in centrosome replication events	41
3) <i>E2F is required for centrosome duplication</i>	42
C - CENTROSOME AND CANCER, DEREGLATION OF THE CENTROSOME CYCLE	42
1) <i>The role of p53 in regulating the centrosome cycle</i>	43
2) <i>BRCA1, a suppressor protein involved in centrosomal functions</i>	43
IV RESEARCH SUBJECT PRESENTATION - AIMS OF THESIS.....	44
MATERIALS AND METHODS	46
I MATERIALS	46
A - CELL CULTURE.....	46
1) <i>Tissue culture cell lines</i>	46
2) <i>Hybridoma culture</i>	47
B - <i>ESCHERICHIA COLI</i> STRAINS USED DURING THIS WORK:	47
C - PEPTIDES	48

D - PRIMERS	48
E - ANTIBODIES.....	49
F - COMMON REAGENTS AND BUFFERS	50
II CELL BIOLOGY TECHNIQUES.....	51
A - TRANSFECTION OF HeLa JW CELLS.	51
B - SYNCHRONISATION OF CELLS.	52
1) Arresting Jurkat cells in S phase	52
2) Arresting HeLa S3 cells in prometaphase.....	52
C - CELL DNA CONTENT ANALYSIS BY FLOW CYTOMETRY	52
D - IMMUNOFLUORESCENCE.....	53
1) Fixation of the sample.....	53
2) Staining of the sample.....	54
3) Mounting and analysis of the sample	54
4) Immunofluorescence on suspension cells.....	54
III BIOCHEMICAL TECHNIQUES.....	55
A - EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS.....	55
B - PURIFICATION OF GST/HIS-TAGGED PROTEIN UNDER DENATURING CONDITIONS.....	56
1) His tagged proteins	56
2) GST tagged proteins.....	57
C - PURIFICATION OF ANTIBODIES	58
1) Affinity purification on a column.....	58
2) Affinity purification on a nitrocellulose membrane	59
3) Affinity purification on protein G.....	59
D - PROTEIN EXTRACTION FROM EUKARYOTIC CELLS.	60
1) Standard protein extraction procedure	60
2) Concentrated extract.....	60
E - DETERMINATION OF PROTEIN CONCENTRATION.....	61
F - IMMUNOBLOTTING	61
1) Gel electrophoresis (SDS-PAGE).....	62
2) Protein transfer on nitrocellulose and antibody incubation	62
3) Immunological detection of protein.....	63
4) Stripping of nitrocellulose membrane	64

G - DOT BLOTTING.....	64
H - PREPARATION OF RECOMBINANT PROTEIN FOR INJECTION INTO RABBITS.	64
I - PURIFICATION OF PORCINE BRAIN TUBULIN	66
J - LABELLING OF TUBULIN WITH RHODAMINE	68
1) <i>Producing polymerisation-competent tubulin</i>	68
2) <i>Labelling of tubulin with rhodamine</i>	69
K - PREPARATION OF CYTOSTATIC FACTOR ARRESTED FROG EGG EXTRACT	70
L - CENTROSOME RELATED TECHNIQUES.....	71
1) <i>Purification of centrosomes</i>	71
2) <i>Aster formation assay</i>	72
3) <i>Stripping of centrosomes</i>	73
4) <i>Immunofluorescence analysis of centrosomal fractions</i>	73
M - SILVER STAINING OF POLYACRYLAMIDE GELS	74
N - SAMPLE PREPARATION FOR MASS SPECTROMETRY ANALYSIS.....	75
IV NUCLEIC ACID TECHNIQUES.....	76
A - EXTRACTION OF PLASMID DNA FROM BACTERIA	76
1) <i>from a small volume of culture (mini-prep)</i>	76
2) <i>from a large volume of culture</i>	76
B - RESTRICTION DIGEST OF DNA.....	77
C - AGAROSE GEL ELECTROPHORESIS.	77
D - PURIFICATION OF DNA FROM AGAROSE GELS.....	78
E - BLUNTING OF 5' AND 3' DNA OVERHANGS	78
F - LIGATION OF DNA	79
G - VECTOR CONSTRUCTIONS.....	79
1) <i>pRSET-MCC₈₂₁ and pRSET-MCC₉₈₇</i>	79
2) <i>Construction of pRSET-HCA66 and pRSET-HCA66₈₇₋₃₆₆</i>	80
3) <i>Construction of pCMV-HCA66myc</i>	80
4) <i>Construction of pRSET-x4.1R</i>	81
5) <i>Construction of GST-x4.1R¹³⁵</i>	81
H - PREPARATION OF <i>E. COLI</i> COMPETENT FOR TRANSFORMATION BY HEAT-SHOCK.....	82
I - TRANSFORMATION OF <i>ESCHERICHIA COLI</i> STRAINS.	82
J - SEQUENCING.....	83
K - POLYMERASE CHAIN REACTIONS.....	84

L - RNA EXTRACTION AND RT-PCR.	84
1) <i>RNA Extraction</i>	84
2) <i>First strand cDNA synthesis</i>	85
RESULTS.....	86
I IDENTIFICATION OF CELL CYCLE-DEPENDENT CHANGES OF THE PCM.....	87
A - PURIFICATION OF CENTROSOMES FROM JURKAT CELLS	87
B - THE FRACTIONS ARE HIGHLY ENRICHED IN CENTROSOMES	90
C - ISOLATION OF "G1" AND "S" CENTROSOMES.....	90
1) <i>A Jurkat cell population enriched in S phase</i>	92
3) <i>Quantitative analysis of the presence of some known proteins at "G1" and "S" centrosomes</i>	94
4) <i>Stripped centrosomes do not nucleate tubulin</i>	96
D - THERE ARE DIFFERENCES IN THE PCM COMPOSITION OF "G1" AND "S" CENTROSOMES.....	100
1) <i>Is septin a centrosomal protein?</i>	104
2) <i>Characterisation of the MCC1 protein</i>	106
3) <i>Characterisation of the HCA66 protein</i>	109
E - PURIFICATION OF CENTROSOMES FROM CELLS IN MITOSISCENTROSOMES	116
II 4.1R A MULTI-FACETED PROTEIN	118
A - IS 4.1R A CENTROSOMAL PROTEIN?	118
B - CHARACTERISATION OF POLYCLONAL ANTIBODIES RAISED AGAINST 4.1R	119
C - A MOUSE POLYCLONAL ANTIBODY RECOGNISING 4.1R.....	123
D - β -TUBULIN AND 4.1 SHARE A STRUCTURAL HOMOLOGY	124
DISCUSSION.....	131
HSP73 IS RECRUITED AT THE CENTROSOME AT THE BEGINNING OF S PHASE	134
A CENTROSOMAL ROLE FOR PYRUVATE KINASE?.....	134
THE PARTICULAR CASE OF SEPTIN.....	135
HCA66, A NOVEL CENTROSOMAL PROTEIN	136
PCM-1 IS RECRUITED TO THE CENTROSOME IN S PHASE	139
4.1R AT THE CENTROSOME?	141

4.1R AND MICROTUBULES.	141
CONCLUSIONS	144
BIBLIOGRAPHY	145
APPENDIX 1: SEQUENCES	173
APPENDIX 2: PUBLICATION	183

List of Figures

Figure 1:	Structure of the centrosome	p4
Figure 2:	Schematic representation of the budding yeast spindle pole body and localisation of its components	p8
Figure 3:	Models of microtubule nucleation by the γ -TuRC	p16
Figure 4:	Schematic representation of 4.1R cDNA and proteins	p22
Figure 5:	An overview of the centrosome cycle	p29
Figure 6:	Schematic representation of the centriole assembly pathway	p31
Figure 7:	Isolation of centrosomes through a sucrose gradient	p88
Figure 8:	Immunoblotting of sucrose gradient fractions	p89
Figure 9:	Jurkat cells arrest in S phase when treated with aphidicolin	p93
Figure 10:	Immunoblot of various centrosomal proteins on isolated centrosomes	p93
Figure 11:	Immunofluorescence of centrosomal proteins	p95
Figure 12:	Stripping of centrosomes with KI	p98
Figure 13:	Stripped centrosomes do not form asters in pure tubulin, but can be reconstituted in frog egg extracts	p99
Figure 14:	There are differences in the soluble fraction of "G1" and "S" centrosomes	p101
Figure 15:	Septin, a centrosomal protein?	p105
Figure 16:	Characterisation of the MCC1 protein	p108
Figure 17:	Sequence comparison between human, mouse, <i>Anopheles gambiae</i> , <i>Drosophila melanogaster</i> , and <i>Saccharomyces cerevisiae</i> HCA66 proteins realised with ClustalX	p110
Figure 18:	Characterisation of the anti HCA66 serum	p111-3
Figure 19:	HCA66 is a centrosomal protein	p115
Figure 20:	4.1R is not detected in the centrosome	p119
Figure 21:	The different polypeptides used to raise antibodies against 4.1R	p120
Figure 22:	Characterisation of the different anti-4.1 polyclonal antibodies	p122
Figure 23:	Characterisation of an anti-4.1R mouse polyclonal antibody	p124
Figure 24:	A serum raised against a 4.1R C-terminal peptide stains the microtubule network	p126
Figure 25:	1782 serum recognises tubulin	p127
Figure 26:	Anti-4.1R antibodies cross-react with β -tubulin	p128
Figure 27:	4.1R peptide and β -tubulin share some homology	p129

List of Tables

Table 1:	Spindle pole components of <i>Saccharomyces cerevisiae</i>	p9
Table 2:	The γ -TuRC composition in different species	p14
Table 3:	Primers used for cloning and sequencing	p48
Table 4:	Antibodies used during this work	p49
Table 5:	Common reagents and buffers	p50
Table 6:	Quantification of the signal of various centrosomal proteins on "G1" centrosomes and "S" centrosomes	p96
Table 7:	Proteins identified by MALDI-tof mass spectrometry	p103
Table 8:	Antibodies raised using different 4.1R antigens	p130

ABSTRACT

The centrosome is the microtubule organising centre of animal cells and is composed of a pair of centrioles surrounded by an electron-dense mass of proteins, called the pericentriolar material (PCM). Several proteins of the PCM exhibit a dynamic association with the centrosome in a cell cycle-dependent manner. The centrosome duplicates during S-phase of the cell cycle and may play an essential role during the transition from G1 to S phase of the cell cycle.

In order to better understand the mechanism of centrosome duplication and the role of the centrosome at the G1/S transition, we decided to investigate the overall changes occurring in the PCM between G1 and S phase by comparing the composition of the PCM at these two cell cycle stages. Therefore, we isolated centrosomes from unsynchronised (mainly in G1) Jurkat cells and from cells arrested in early S phase. Centrosomes were further fractionated by salt extraction with potassium iodide into soluble and insoluble material. Comparative gel electrophoresis of the soluble fractions in G1 and S phase allowed us to detect several differences in the protein composition. Using MALDI-tof mass spectrometry, we identified eight different proteins specifically accumulating in either phase. Among those accumulating in S-phase, some were known proteins whereas others were novel uncharacterised proteins. Thus, our results led to the identification of new proteins potentially recruited to the centrosome in S phase where they may play a specific role in cell cycle-dependent centrosome functions. We started the study of HCA66, one of the novel proteins. We cloned its full-length cDNA by RT-PCR from Jurkat cells, expressed a recombinant portion of the protein in bacteria and raised a polyclonal antibody. A protein of ~60kDa corresponding to the predicted molecular weight of HCA66 was enriched in the PCM from S phase centrosomes. Thus, we laid the basis for further investigation on the function of HCA66 at the centrosome.

The protein 4.1R, previously characterised as a component of the plasma membrane skeleton, is also localised at the centrosome. We investigated the role of this protein at the centrosome and found that the carboxy terminus of 4.1R bears similarities to a short motif in β -tubulin.

INTRODUCTION

Mitosis is the process by which a cell divides, forming two identical daughter cells. The correct segregation of chromosomes is essential to ensure the propagation of cells and species, as errors of segregation lead to aneuploidy and promote tumor development and progression. The mitotic spindle, a complex superstructure composed of microtubules, associated proteins and a pair of centrosomes, mediates chromosome segregation. The centrosome, named after its central position in the cell, was first discovered more than a century ago and thought to be "the organ of cell division *par excellence*" (Wilson 1895). The revolution of molecular biology, the use of genetics, biochemistry and advanced microscopy led to a better understanding of centrosome ultrastructure, to the discovery of many centrosomal proteins and shed light on other important functions of the centrosome.

I Structure of centrosomes and spindle pole bodies

Microtubules organising centres (MTOCs) are a morphologically diverse group of organelles which fulfil related functions. They are basal bodies in green algae, the centrosome in higher eukaryotes, and spindle pole bodies (SPB) in yeast. We will focus

on the biological role of the centrosome and its ultra-structure and will give a brief overview of the current knowledge of spindle pole bodies.

A – Centrosome structure

The centrosome is a very small membrane free organelle ($1-2 \mu\text{m}^3$) sitting near the centre of the cell, close to the nucleus to which it is anchored. In most animal cells, centrosomes consist of two centrioles associated with a cloud of electron-dense material called the pericentriolar material (PCM) (Figure 1A, 1B). The two centrioles are close together, generally in an orthogonal orientation, and are linked to each other by filamentous and amorphous material (Bornens *et al.* 1987). The PCM is an interconnected meshwork of $\sim 12-15$ nm fibres and protein aggregates forming a lattice, known as the centromatrix. The centromatrix is an organised structure tightly associated with the centriole which directs the recruitment of other centrosomal components such as the γ -tubulin ring complex, responsible for microtubule nucleation.

A centriole exhibits a barrel shape of ~ 400 nm in length with its sides made of microtubules. The arrangement of these microtubules is complex. At the extremity where the two centrioles are the closest (proximal end), the barrel wall consists of nine triplets of microtubules (tubules A, B, C). This organisation changes towards the distal end (where the two centrioles are the farthest apart), where the wall of the centriole consists of nine doublets (tubules A, B) (Figure 1C).

The two centrioles are not structurally identical. One, called the mother centriole, has sub-distal and distal appendages at its distal end. These appendages are absent from the other centriole, which is called the daughter centriole (Figure 1C). Electron

microscopy studies on isolated centrosomes revealed that distal appendages are oriented with a nine-fold symmetry around the centriole and appear as rigid sticks probably attached to the tubule B of the centrioles (Paintrand *et al.* 1992). The sub-distal appendages show more variability in their number and distribution than the distal appendages. They have a broad base allowing them to interact with two sets of adjacent microtubules (Vorobjev and Chentsov 1980). Furthermore, sub-distal appendages disappear in the G2 phase of the cell cycle in mammalian somatic cells and reform in early G1 on the mother centriole (Vorobjev and Chentsov Yu 1982).

The exact function of these appendages remains unclear. The tip of the sub-distal appendages has been described as a nucleation site and/or a binding site for microtubules in PE and PtK2 cells and also *in vitro* from isolated centrosomes (Vorobjev and Chentsov Yu 1982; De Brabander *et al.* 1986; Chretien *et al.* 1997). Ninein, a candidate for microtubule anchoring at the centrosome, was recently located at the sub-distal appendages, suggesting a role of these appendages in the anchoring mechanism (Mogensen *et al.* 2000).

Not all centrioles are as complex in their structure. For instance, centrioles in *Drosophila melanogaster* embryo are composed of nine singles or doublets of microtubules arranged in a ring and connected to the centre via spokes (Moritz *et al.* 1995; Callaini *et al.* 1997). This simpler centriole structure might be a result of rapid mitosis occurring in the embryo, therefore limiting the available time for building a more complex structure (Moritz *et al.* 1995).

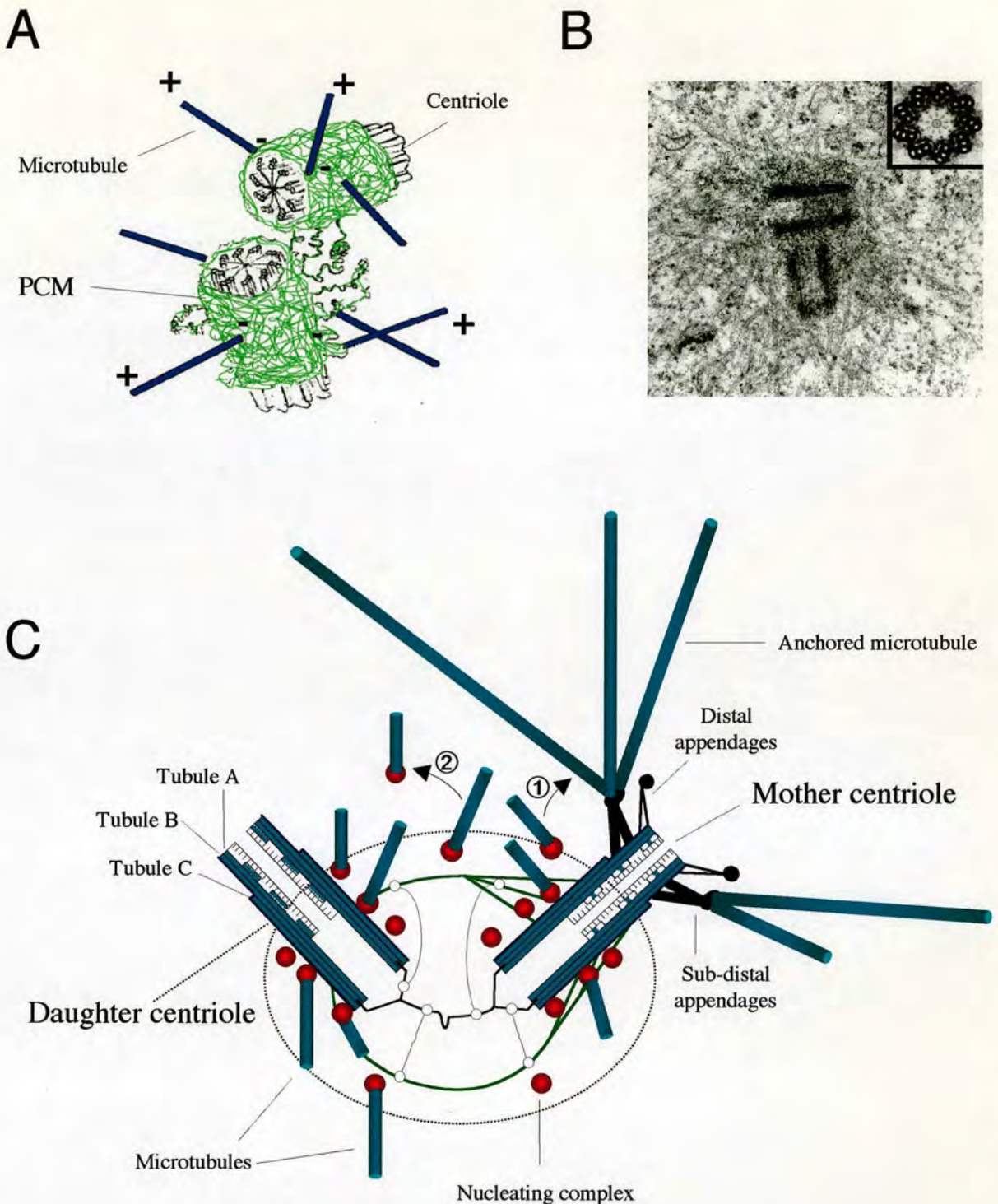


Figure 1: Structure of the centrosome.

A, Schematic representation of a centrosome. Pericentriolar material is in green and microtubules are in light blue. **B**, Electron micrograph showing a longitudinal section of the two centrioles surrounded by the pericentriolar material. Top right shows a cross-section of a centriole (from de Harven 1994). **C**, Schematic representation of a centrosome model (adapted from Bornens 2002). Mother and daughter centrioles are linked by microtubule-binding proteins. They are able to bind proximal minus end of centrioles (black) or centriole walls through interaction with polyglutamylated tubulin (green and blue respectively). These two types of proteins are linked together by other proteins (grey) forming the fully assembled matrix (black dotted line). Microtubules (blue cylinder) are nucleated by nucleating complexes (red) in the vicinity of both centrioles. After nucleation, microtubules are either anchored by the mother centriole [①] or released into the cytoplasm [②].

Centrioles are structurally similar to basal bodies. In some organisms, such as *Chlamydomonas reinhardtii*, the same structure acts as a basal body in interphase and a centriole in mitosis (Coss 1974). Moreover, in most animals, after fertilisation the basal body of the sperm becomes the centriole of the egg's centrosome (see Schatten 1994 for review).

In some cell types, the mother centriole can elongate the microtubules of its distal end to produce the primary cilium (see Wheatley *et al.* 1996 for review).

Centrioles are not responsible for microtubule nucleation since cells from higher plants or some meiotic cells, lacking centrioles, are able to form acentriolar MTOCs which can nucleate and organise microtubules. Bobinnec and co-workers demonstrated that centrioles are more likely to play a role in centrosomal organisation (Bobinnec *et al.* 1998). Microinjection of an antibody against glutamylated tubulin (found mainly at the centriole) results in centriole disassembly and PCM disorganisation. Moreover, after removal of this antibody the PCM reformed around the centrioles. Furthermore, SAS-4, a centriolar protein recently identified in *Caenorhabditis elegans*, has been implicated in the control of centrosome size: the amount of PCM recruited by the centrioles is proportional to the quantity of SAS-4 present (Kirkham *et al.* 2003). These data suggest that centrioles are required for the proper organisation of the centrosome by focussing the PCM.

B – Spindle pole body: an overview

The spindle pole body, the yeast equivalent of the centrosome, is much smaller than its higher eukaryote counterpart ($0.01 \mu\text{m}^3$). Electron microscopy, yeast genetics and biochemical approaches on purified SPBs made the *Saccharomyces cerevisiae*'s SPB the most extensively MTOC characterised to date.

1) Ultrastructure of the spindle pole body of *Saccharomyces cerevisiae*

The spindle pole body of *Saccharomyces cerevisiae* is a multilayered cylindrical entity anchored in the nuclear envelope throughout the cell cycle (Figure 2). Electron microscopy analysis revealed that the SPB consists of an outer plaque facing the cytoplasm, a central plaque, and an inner plaque directed towards the nucleus (see Adams and Kilmartin 2000 for review). Moreover, a structure called half-bridge is adjacent to the SPB. The half bridge is a one-sided extension of the central plaque layered on top of the nuclear envelope that functions in the cell cycle dependent duplication of the SPB. Other structures (namely IL1, IL2, IP1 and IP2) were detected on isolated SPBs using cryo-electron microscopy and image processing (Bullitt *et al.* 1997; see Helfant 2002 for a review). IL1 and IL2 (intermediate lines 1, 2) are two thin layers which separate the central and the outer plaques. IP1 (inner plaque 1), a beaded layer, and IP2 (inner plaque 2), containing the tips of microtubules, form the inner plaque. The spindle pole body organises two arrays of microtubules: the cytoplasmic microtubules, which originate from the cytoplasmic side of

the SPB and are directed towards the cortex of the cell and the nuclear microtubules, which originate from the nuclear side of the SPB and are directed towards the nucleoplasm. The cytoplasmic microtubules are implicated in the positioning, alignment and movement of the nucleus. The nuclear microtubules are implicated in SPB separation, spindle formation and chromosome segregation.

2) Molecular composition of the SPB

Several strategies were used to identify the components of the SPB. Rout and Kilmartin raised monoclonal antibodies against partially purified spindle pole bodies and identified several SPB components including Spc97p and Spc98p, two proteins of the yeast Tub4p complex (see below and section II-B-1, Rout and Kilmartin 1990). More recently 1D-gel electrophoresis of purified spindle pole bodies followed by mass spectrometry, was used to identify additional components (Wigge *et al.* 1998). Other spindle pole components, such as Cdc31p or Kar1p, were identified using genetic analyses (Byers 1981a; Byers 1981b; Conde and Fink 1976; Schild *et al.* 1981, see Table 1 for a list of spindle pole component of *Saccharomyces cerevisiae*).

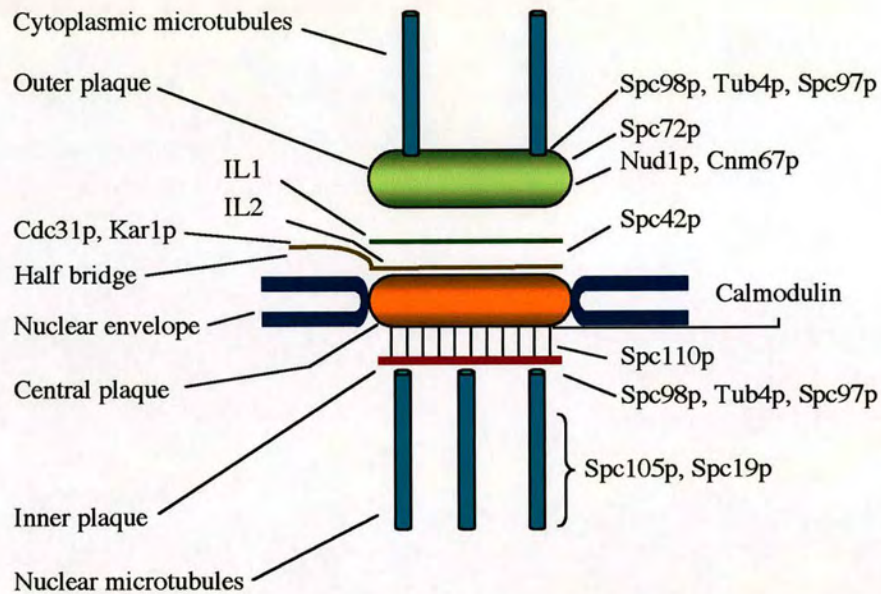


Figure 2: Schematic representation of the budding yeast spindle pole body and localisation of its components. Ndc80p, and Spc's 105p, 34p, 25p, 24p, 19p are presumably associated with the spindle. IL1 and IL2: intermediate lines 1 and 2. Adapted from Wigge et al., 1998.

Protein	MW (kDa)	Location	Function
Stu1p	174	Pole and spindle	Mitotic spindle assembly
Spc110p	110	SPB CP/OP	Anchor site for the Tub4 complex at IP
Spc105p	105	Pole	
Stu2p	100	SPB OP/ spindle pole	Mitotic spindle elongation in anaphase
Spc98p	98	SPB OP/IP	Associates with Tub4 complex as part of MT attachment site of SPB
Spc97p	97	SPB OP/IP	Associates with Tub4 complex as part of MT attachment site of SPB
Nud1p	94	SBP OP	Nuclear division
Ndc1p	74	SPB CP	Nuclear envelope insertion of the SPB
Spc72p	70	SPB OP	Docking protein for Tub4p complex at the OP
Cnm67p	67	SPB OP	Nuclear migration
Tub4p	52	SPB OP	Part of Tub4p complex, MT nucleation
Kar1p	51	SPB HB	SPB duplication
Bbp1p	45	SPB CP	Mitotic cell cycle
Spc34p	34	Pole and spindle	
Spc29p	29	SPB CP	SPB duplication
Spc19p	19	Pole and spindle	
Cdc31p	19	SPB HB	Centrin involved in SPB duplication
Cmd1p	16	SPB CP	Nuclear division, chromosome maintenance.

Table 1: Spindle pole components of *Saccharomyces cerevisiae*. CP: central plaque, HB: half-bridge, IP inner plaque, MT: microtubules, MW: molecular weight, OP: outer plaque, SPB: spindle pole body. (adapted from Helfant 2002).

3) Tub4p and the Tub4p complex

The γ -tubulin gene, named *TUB4*, was identified by the yeast genome-sequencing project (Goffeau *et al.* 1996). The protein was identified at the inner and outer plaques of the SPB. Analysis of Tub4p-depleted cells and *TUB4* mutants demonstrated the importance of this protein in microtubule nucleation (Sobel and Snyder 1995; Marschall *et al.* 1996).

Tub4p associates with Spc97p and Spc98p in the yeast Tub4p complex (also known as the γ -TuRC (γ -tubulin ring complex)). This complex is localised in the inner and outer plaques of the SPB. It is anchored to these substructures via the binding of Spc97p and Spc98p to the SPB proteins Spc72p and Spc110p.

4) The role of the Spc110p protein

Spc110p was originally identified by Rout and Killmartin (Rout and Killmartin 1990). The protein localises at the nuclear face of the SPB and its amino- and carboxy-terminal ends are anchored at the inner and central plaque respectively (Spang *et al.* 1996; Sundberg *et al.* 1996). Besides a role of spacer protein between the inner and outer plaques, Spc110p has been described as a Tub4p complex-binding protein. It interacts with Spc97p and Spc98p, but not with Tub4p, anchoring the Tub4p complex to the inner plaque.

5) Spc72p anchors the Tub4p complex at the outer plaque

Whereas Spc110p anchors the Tub4p complex at the inner plaque, another protein called Spc72p, identified as a SPB component interacting with Spc97p and Spc98p, targets it to the outer plaque (Fields and Song 1989). More recently, a Spc72p-GFP fusion protein was identified at the half-bridge in early G1 cells (Adams and Killmartin 1999). It is consistent with the notion that cytoplasmic microtubules are initiated from the half-bridge

during this time, implying that the Tub4p complex is also present at the half bridge during G1 (Byers and Goetsch 1975; Knop and Schiebel 1998). The anchoring of Spc72p to the half-bridge depends on Kar1p whereas its anchoring to the outer plaque depends on Nud1p. The ability of the SPB to nucleate microtubules from different regions at different times in the cell cycle (half bridge in early G1, outer plaque in S) could be linked to variation of Spc72p localisation. Such variations have previously been described for G1 and mitosis. In mitosis Spc72p was predominantly located at the outer plaque, whereas in early G1 it was located at the half bridge (Pereira *et al.* 1999; Adams and Kilmartin 1999).

II Biological roles of the centrosome

For a long time, centrosomes were considered as the microtubule organising centre of animal cells. In recent years, new biological roles of the centrosome have been discovered; in addition to microtubule nucleation, it is involved in spindle positioning and cell cycle progression.

A - The centrosome as a microtubule organising centre

Microtubules are hollow tubes of ~25 nm in diameter consisting of 13 protofilaments arranged in a cylinder. A protofilament is composed of α and β -tubulin heterodimers organised in a head to tail manner. *In vitro*, studies have shown that

microtubules are dynamic structures, exchanging their tubulin with the soluble pool of tubulin. Two mechanisms have been proposed to explain these dynamics: treadmilling and dynamic instability. Treadmilling relies on the fact that one microtubule end (termed the plus end) predominantly incorporates tubulin dimers, whereas the other end (termed the minus end) predominantly loses them. Treadmilling results in an apparent movement of the microtubule or, when the microtubule is stationary, in an apparent flux of tubulin molecules travelling through the microtubule (Margolis and Wilson 1981). However, the treadmilling model is inconsistent with measured rates of microtubule dynamics and fails to explain the observation that microtubule polymers can fluctuate in length (sometimes to zero), at equilibrium condition. The dynamic instability model describes microtubule dynamics as a result of stochastic transitions between assembly and disassembly of microtubule ends (Mitchison and Kirschner 1984b). Transitions from assembly to a sudden disassembly are named "catastrophe", whereas transitions from disassembly to assembly are referred to as "rescue".

It has been noted that microtubules polymerised *in vitro*, have a variable number of filaments, whereas *in vivo* the number of filaments is constant, suggesting the presence of a template for microtubule assembly. *In vivo*, the cellular concentration of tubulin is below the concentration allowing self-assembly and nucleation occurs mainly from the centrosome by plus end addition of tubulin subunits (McIntosh and Euteneuer 1984; Mitchison and Kirschner 1984a; Mitchison and Kirschner 1984b). *In vivo*, many microtubules minus ends are capped by the centrosome preventing "catastrophe" events and microtubule minus end disassembly, hence limiting the phenomenon to the plus ends (Kirschner and Mitchison 1986).

One role of the centrosome is to nucleate and organise the interphase microtubule network and the mitotic spindle. However, an interphase microtubule array can be formed

in the absence of centrosomes (Rodionov and Borisy 1997). Microtubules are nucleated "randomly" within the cytoplasm and are organised in an array by the action of molecular motor proteins such as cytoplasmic dynein (Rodionov and Borisy 1997). The centrosome is the main site of microtubule nucleation in the cell and, when present, act in a dominant way. If microtubules are artificially depolymerised using drugs or cold treatment, they grow back from the centrosome when the drug is washed out or the cells warmed up. The nucleation activity of the centrosome is located in the PCM; centrioles do not directly participate in the nucleation of cytoplasmic microtubules since they are unable to nucleate microtubules *in vitro* (Gould and Borisy 1977).

B - The role of γ -tubulin and the γ -tubulin ring complex in microtubule nucleation

γ -Tubulin was first identified in the fungus *Aspergillus nidulans* as a result of a genetic screen designed to identify proteins that interact with β -tubulin (Oakley and Oakley 1989). γ -Tubulin is 30% identical to α - and β -tubulin. It has been described in a wide variety of organisms and is likely to be present in all eukaryotes. γ -Tubulin is present in the pericentriolar material of centrosomes where it plays an important role in nucleation of microtubules (Stearns *et al.* 1991; Zheng *et al.* 1991; Moritz *et al.* 1998).

1) The γ -tubulin ring complex

γ -Tubulin and other proteins called γ -tubulin ring proteins (grips) assemble in a 2.2×10^6 Da complex seen as an open lock washer-shaped ring of 25 nm (the same diameter as microtubules) covered by a cap on one face. The complex is found at the minus end of centrosomal microtubules, and in the cytoplasm (Stearns and Kirschner 1994). The complex, called the γ -tubulin ring complex (γ -TuRC), varies in size and complexity between species. The γ -TuRC composition is conserved among higher eukaryotes (Zheng *et al.* 1995). *Drosophila* γ -TuRC contains γ -tubulin, two high molecular weight proteins (Dgrip163 and Dgrip128), two proteins of a molecular weight near 100 kDa (Dgrip91 and Dgrip84) and a group of three or four proteins with molecular masses around 75 kDa (Dgrip75s) ADDIN ENRfu (Oegema *et al.* 1999, see Table 2 for composition of γ -TuRC in *Xenopus laevis* and mammals).

<i>Drosophila Melanogaster</i>	<i>Xenopus laevis</i>	Mammals
Dgrip163	Xgrip210 (Xgrip195)	GCP6
Dgrip128	Xgrip133	GCP5
Dgrip91	Xgrip109	GCP3
Dgrip84	Xgrip104	GCP2
Dgrip75s	Xgrip75s	GCP4
γ -tubulin	γ -tubulin	γ -tubulin (GCP1)

Table 2: The γ -TuRC composition in different species (Zheng *et al.* 1995; Murphy *et al.* 1998; Murphy *et al.* 2001)

The molecular mechanism by which the γ -TuRC nucleates microtubules is subject to controversy. Two different models have been proposed: the template and the protofilament models.

In the first model, supported by recent publications (Keating and Borisy 2000; Moritz *et al.* 2000; Wiese and Zheng 2000), the γ -TuRCs would act as template at the

minus end of the microtubule, initiating each of the 13 protofilaments through longitudinal contacts with the γ -tubulin subunits. In this model, each of the 13 protofilaments is bound to one of the γ -tubulin subunits in the γ -TuRC, allowing capping of the microtubule and blocking assembly and disassembly at the minus end (Figure 3A).

In the protofilament model, γ -TuRC provides a short segment of preformed protofilament, nucleating a second protofilament by lateral contact to α -tubulin and/or β -tubulin (Figure 3B, (Erickson and Stoffler 1996; Erickson 2000).

2) The γ -tubulin small complex

In addition to γ -TuRC, a smaller complex containing γ -tubulin was found in several organisms such as *Drosophila melanogaster* or *Saccharomyces cerevisiae* (Knop *et al.* 1997; Moritz *et al.* 1998; Oegema *et al.* 1999). This γ -tubulin small complex (γ -TuSC) consists of γ -tubulin, Dgrip84 (Spc97/GCP2) and Dgrip91 (Spc98/GCP3), and is likely to be a structural core subunit of the γ -TuRC (Moritz *et al.* 1998). *In vitro* experiments, using isolated *Drosophila* γ -TuSC, showed that the complex can nucleate microtubules, although less efficiently than the γ -TuRC (Oegema *et al.* 1999). Cryoelectron microscopy analysis suggested that the γ -TuRC contains 6 γ -TuSC forming the ring wall of the γ -TuRC (Oegema *et al.* 1999; Wiese and Zheng 1999 for review). The cap structure is made of the non γ -TuSC sub-units Dgrips163, 128 and 75s (Figure 3, Keating and Boris 2000; Moritz *et al.* 2000).

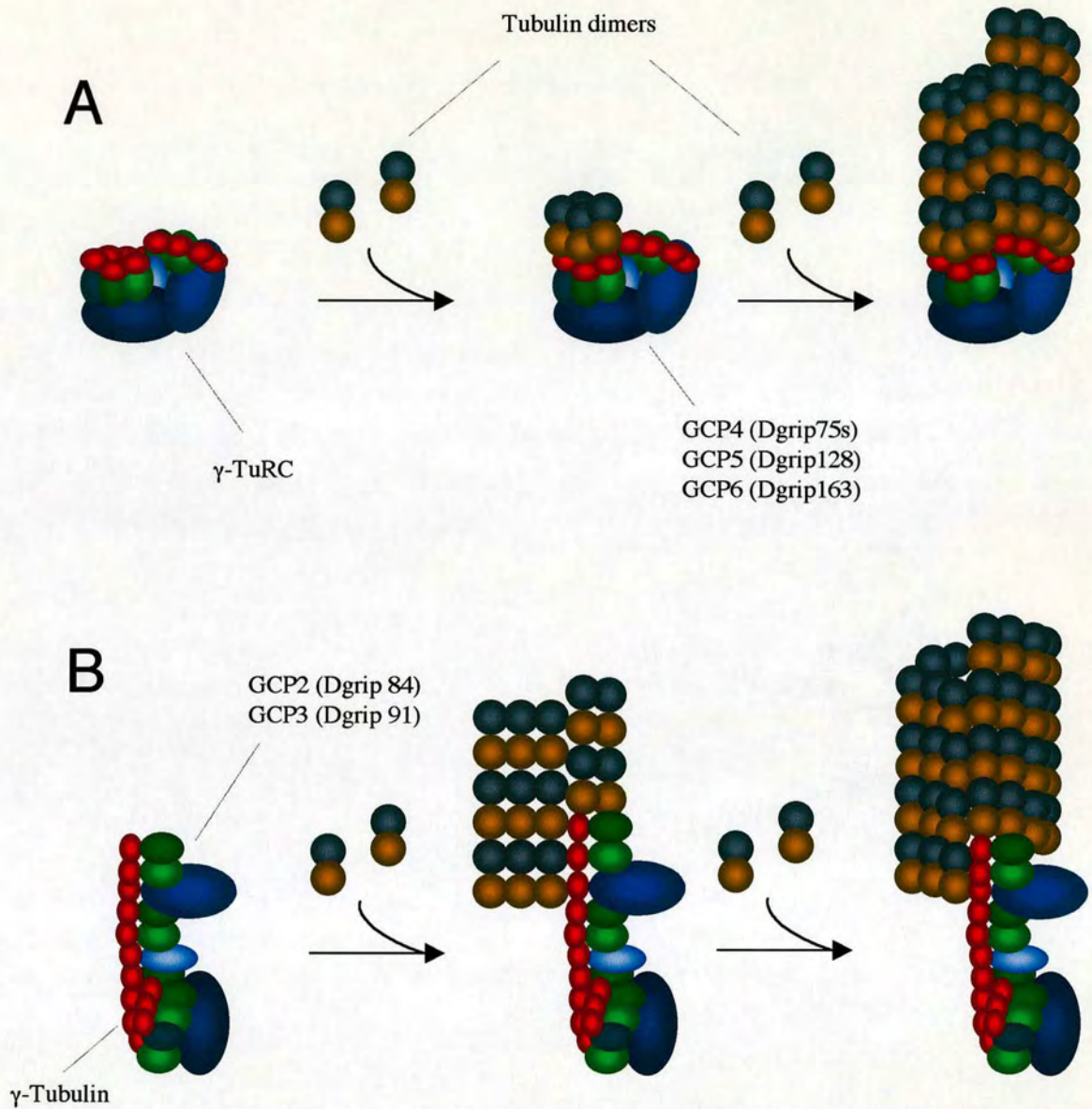


Figure 3: Models of microtubule nucleation by the γ -TuRC.

A, In the template model the γ -TuRC mimics the end of a microtubule where tubulin dimers bind and a microtubule assembles. **B**, In the protofilament model tubulin dimers bind to the γ -TuRC creating a sheet which grows and curls to form the microtubule (adapted from Job *et al.* 2003).

3) How is the γ -TuRC recruited to and anchored at the centrosome?

The way γ -TuRCs are recruited and anchored at the centrosome is not fully understood. In budding yeast, Spc110p interacts with Spc98p/GCP3 and Spc97p/GCP2 and mediates the attachment of the yeast γ -TuRC to the spindle pole body (Knop and Schiebel 1997). In mammalian cells, where the γ -TuRC is more complex, pericentrin has been implicated in the recruitment of γ -TuRC to the centrosome possibly through interactions with GCP2 and GCP3 (Dictenberg et al. 1998; Doxsey 2001). Moreover, pericentrin B, a larger splice variant of pericentrin, shares homology with the calmodulin domain of Spc110p and might be its mammalian counterpart, suggesting that pericentrin B plays a role in γ -TuRC anchoring (Flory *et al.* 2000). Some grip proteins such as Xgrip210 (Dgrip163/GCP6) in *Xenopus* have also been involved in the anchoring of the γ -TuRC to the centrosome though the mechanism has not yet been solved and might involve other proteins (Zhang *et al.* 2000). For instance, in *Drosophila* *Asp* (abnormal spindle) mutants, γ -tubulin does not concentrate on centrosomes but is found dispersed throughout the spindle, supporting the notion that *Asp* is involved in the recruitment of γ -tubulin at the centrosome (Donaldson *et al.* 2001).

C - Anchoring microtubules to the centrosome

In some cells, such as monocytes or fibroblasts, most microtubules originate from the centrosome whereas in others, such as neurons or skeletal muscle cells, most microtubules are not anchored at the centrosome. This raises the question of how some microtubules are anchored at the centrosome, whereas others are free.

Epithelial cells have anchoring sites at their apical domain which bind microtubule minus ends (Mogensen *et al.* 1997). These sites lack centrosomal proteins involved in microtubule nucleation (i.e γ -tubulin, pericentrin), although they contain ninein, a protein associated with the sub-distal appendages of the mother centriole, demonstrating that nucleation and anchoring activities of the centrosome do not depend on the same proteins (Mogensen *et al.* 2000). These appendages act as microtubule-anchoring sites, suggesting that ninein is involved in microtubule anchoring to the centrosome (Chretien *et al.* 1997; Mogensen 1999; Mogensen *et al.* 2000). Although both centrioles are capable of nucleating microtubules, only those nucleated by the mother centriole stay anchored due to the presence of ninein. The molecular mechanism of the transfer of microtubules is unknown. An analogy can be drawn with the nucleation of microtubules in budding yeast and the shuttling of Spc72p. A similar protein could cap microtubules minus ends and interact with proteins of the sub-distal appendages such as ninein therefore promoting microtubule anchoring. The current model by M. Bornens' group proposes that microtubules nucleated by the daughter centriole are either anchored by the mother centriole, or released into the cytoplasm depending on the intra-centriolar distance (Figure 1C, Piel *et al.* 2000; Bornens 2002). If the distance is short enough, the microtubule will be transferred to the mother centriole, otherwise it will be released into the cytoplasm. The motor protein dynactin has also been implicated in the binding of microtubules to the centrosome, though it is not

currently known if dynactin is directly involved in this process, or if it transports anchoring molecules to the centrosome (Quintyne *et al.* 1999).

Evidence suggests that microtubule release from the centrosome is an active mechanism (Ahmad *et al.* 1999). This activity is thought to be mediated by katanin, a protein with severing activity on microtubules both *in vitro* and *in vivo* (McNally and Vale 1993; Hartman *et al.* 1998; Ahmad *et al.* 1999). The release of microtubules could also be the result of an activity mediated by the Kin1 subfamily of kinesin, which destabilises microtubules minus ends (Desai *et al.* 1999).

D - The role of the centrosome in the formation and positioning of the mitotic spindle

1) The centrosome is dispensable for spindle assembly

For a long time, it has been thought that centrosomes were defining the spindle poles and were required for the establishment of a functional bipolar spindle. However, work performed *in vitro* using cytoplasmic extracts from *Xenopus* eggs clearly showed that a functional bipolar spindle could be established around chromatin-coated beads without centrosomes (Heald *et al.* 1996). It has been proposed that Ran-GTP causes the release of spindle pole proteins from a complex with importin α and β thereby promoting nucleation and stabilisation of microtubules (Gruss *et al.* 2001; Nachury *et al.* 2001; Wiese *et al.* 2001).

Ran-GTP activity is mediated through RCC-1, a guanine nucleotide exchange factor associated with the chromatin (Ohtsubo *et al.* 1989). Microtubule motor proteins, including cytoplasmic dynein and the BimC motor proteins, are also involved in bipolar spindle assembly (Sharp *et al.* 1999; Sharp *et al.* 2000). These proteins act together to establish a functional bipolar spindle. Ran-GTP also increases microtubule polymerisation through an, as yet, unknown mechanism (Kahana and Cleveland 1999).

Acentrosomal spindles are also formed in higher plants, in some mammalian oocytes and in theacentrosomal *Drosophila* cell line 1182-4 (Debec *et al.* 1995). Moreover, when centrosomes are artificially removed from CV-1 and BSC-1 cells, they are still able to form a bipolar spindle (Maniotis and Schliwa 1991; Khodjakov *et al.* 2000; Hinchcliffe *et al.* 2001). Furthermore, centrosomes can be artificially removed from the spindle pole without affecting focusing of microtubules (Nicklas *et al.* 1989).

This raises the question why cells have centrosomes at their spindle poles if they are not required for the formation of the mitotic spindle. One view is that it ensures each daughter cell inherits one copy of the organelle so that other essential functions performed by the centrosome can be fulfilled. When present, the centrosome acts in a dominant way in the nucleation of microtubules and the organisation of a bipolar spindle, and the cell is able to build a bipolar spindle in a faster time than inacentrosomal cells, where no pre-formed organising centres are present. The number of centrosomes in a cell at the onset of mitosis determines the number of spindle poles. Cells containing a single centrosome or an unseparated doublet form a monopolar spindle while cells containing more than two centrosomes form multipolar spindles (Bajer 1982; Sawin *et al.* 1992; Mayer *et al.* 1999; Heneen 1975; Sluder and Rieder 1985).

Thus, vertebrate cells have a redundant pathway of establishing a bipolar spindle and there are components not related to the centrosome stabilising microtubule minus ends. The

nuclear and mitotic apparatus (NuMA) protein is able to stabilise and focus microtubules through a direct interaction (Merdes *et al.* 1996; Haren and Merdes 2002). In *Xenopus laevis* egg extracts, NuMA is required for assembly of the spindle pole and to maintain microtubule minus-end focusing (Merdes *et al.* 1996). NuMA can oligomerize and bind several microtubules inducing their tethering. Other proteins, such as 4.1R, may also be involved in the formation and the maintenance of the spindle pole.

2) The 4.1R protein is present at the centrosome

The 4.1R proteins belong to a large protein family. These proteins arise from a single gene by alternative splicing, usage of three translation initiation sites and post-translational modifications (Figure 4). These isoforms have apparent molecular weights ranging from 30 to 210 kDa and are widely expressed in many tissues (Granger and Lazarides 1984; Anderson *et al.* 1988)

The 80kDa protein 4.1R (4.1R⁸⁰) is a component of the membrane skeleton first described on the basis of its electrophoretic mobility in erythrocyte lysates (Ungewickell *et al.* 1979). The 4.1R¹³⁵ isoform is generated by usage of an upstream translation initiation codon. The protein contains an additional 209 amino-acid domain located in the amino-terminal part of the protein (compared to 4.1R⁸⁰). 4.1R proteins have been located in several sub-cellular compartments where they have different biological roles.

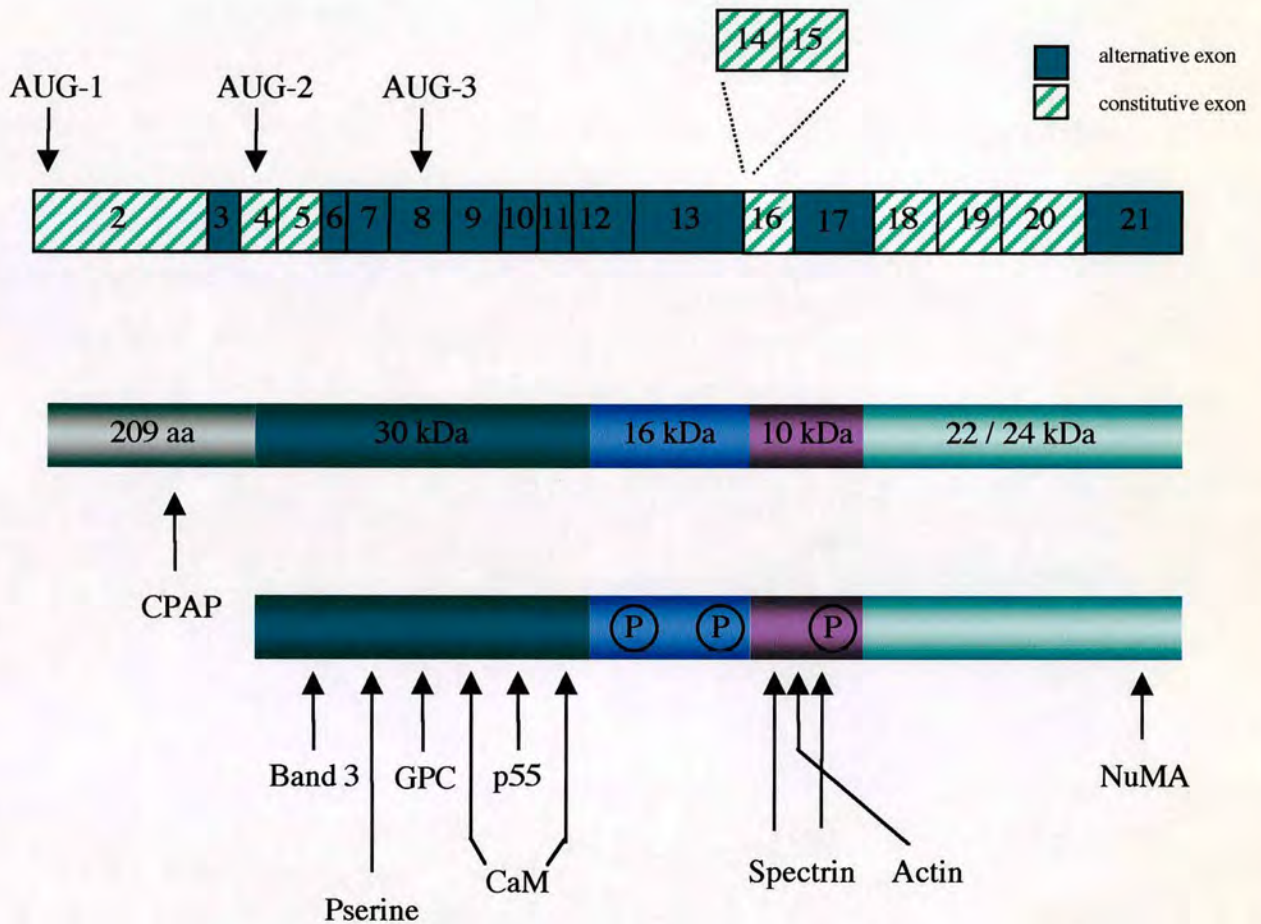


Figure 4 : Schematic representation of 4.1R cDNA and proteins.

The different domains of 4.1R protein (30kDa, 16kDa, 10kDa, 22-24kDa) were defined by partial chymotryptic digestion and functional analysis (Leto *et al.* 1984). Corresponding exons are shown. The amino-terminal 30kDa domain is encoded by exons 5-12. It binds transmembrane proteins such as band 3, phosphatidyl serine (Pserine), Glycophorine C (GPC), p55 and calmodulin (CaM) (Hemming *et al.* 1994; Pasternack *et al.* 1985). No specific binding sites were mapped in the 16kDa domain, encoded by exons 12-13. The 10kDa domain, known as the SABD (Spectrin/Actin-binding domain) encoded by exons 14-17, has a binding site for spectrin and actin and, *in vitro*, plays a critical role in nuclear assembly (Correas *et al.* 1986a; Correas *et al.* 1986b). The 22-24kDa domain (CTD: Carboxy-terminal domain), encoded by exons 17-21, interacts with NuMA (Mattagajasingh *et al.* 1999) and is involved in nuclear assembly as determined by *in vitro* experiments (Krauss *et al.* 2002). The additional 209aa domain present in 4.1R¹³⁵ contains a binding site for Centrosomal P4.1-Associated Protein (CPAP). Phosphorylation sites for PKA or PKC are also shown (P circled). Adapted from Takakuwa (2000).

4.1R proteins are components of the centrosome, forming part of the pericentriolar material (Krauss *et al.* 1997b). More recently, CPAP (Centrosomal P4.1-Associated Protein), a newly identified centrosomal protein associated with the γ -tubulin complex, was shown to interact with 4.1R¹³⁵ at the centrosome via its 209 amino acid domain (Hung *et al.* 2000). It has been postulated that 4.1R could act as an adapter to anchor the CPAP/ γ -tubulin complex at the centrosome (Hung *et al.* 2000). However, the role of the protein at the centrosome remains to be clarified. 4.1R is also present at the spindle pole where it interacts with NuMA, though a direct interaction with NuMA does not seem required for assembly of the protein at the spindle pole (Mattagajasingh *et al.* 1999; Delhommeau *et al.* 2002).

It has been suggested that 4.1R could be at the vicinity of the centrosome rather than a *bona fide* centrosomal component where it would associate to CPAP and to a yet unidentified-complex through its carboxy-terminal domain. This association would be required for 4.1R to localise to the centrosome and the spindle pole (Hung *et al.* 2000). 4.1R could be a centrosomal component playing a structural role in the spindle pole architecture. It could promote or facilitate docking of NuMA at the spindle poles during mitosis ensuring a connection between the centrosome and the spindle pole. Alternatively, 4.1R could mediate the interaction between the actin related protein Arp-1 and NuMA, therefore establishing binding of NuMA to the dynein/dynactin complex during mitosis, as suggested by Clark and Meyer (Clark and Meyer 1999).

It is not currently known whether the the alternate pathway of spindle formation based on self-organisation of microtubules and motor proteins is active in cells having

centrosomes or whether it works only when the centrosome is absent. This redundancy, if it exists, could be a way for the cell to increase the fidelity of chromosome segregation, which is *in fine* the role of the mitotic spindle. Although centrosomes might not be essential for segregating chromosomes they have other important roles in mitosis. They nucleate astral microtubules, which play a role in determining the position of the cleavage furrow through spindle positioning by interaction with the dynein motor protein (Busson *et al.* 1998; O'Connell 2000). Therefore, cells without centrosomes lack the ability to reposition the spindle as their shape changes during mitosis, leading to defects in cytokinesis as discussed below.

E - Role of the centrosome during cytokinesis

Recent studies have shown that the centrosome is required for progression through the cell cycle (Hinchcliffe *et al.* 2001; Khodjakov and Rieder 2001). When the centrosome is artificially removed, either by laser ablation or using a micro-needle, nearly half the cells fail to complete cytokinesis. The two daughter cells remain attached together by intracellular bridges containing chromatin or exit mitosis leading to the formation of binucleated cells (Khodjakov and Rieder 2001). These observations suggest that the centrosome plays a role in cytokinesis, though it is not required *per se*, as half the cells complete a normal mitosis. The cells lacking centrosomes form an acentrosomal spindle and do not have astral microtubules. It often leads to an abnormal positioning of the spindle at the onset of anaphase (the long axis of the spindle is perpendicular to the long axis of the cell) and to failure of cytokinesis (Khodjakov and Rieder 2001). The central nervous system of *Drosophila melanogaster* larvae is an example of the

centrosome's role in spindle positioning, where neuroblasts divide asymmetrically to form another neuroblast and a smaller ganglion cell. This asymmetry is generated by the progressive disappearance of one centrosome and its astral microtubule triggering a shift of the spindle towards one side of the cell (Kaltschmidt *et al.* 2000). However, other studies using fly mutants showed that asymmetric divisions can occur without astral microtubules, suggesting the existence of a redundant process ensuring proper division in their absence (Bonaccorsi *et al.* 2000; Megraw *et al.* 2001). Thus, centrosomes might not play a direct role in cytokinesis, they rather organise and maintain the astral microtubule array to ensure proper cytokinesis.

Another possibility would be that the centrosome activates the final stage of mitosis or releases the cell from an inhibitory mechanism such as a checkpoint. Such a checkpoint has been described in budding yeast. The cell remains in cytokinesis until the spindle pole body moves into the bud bringing together Tem1-GDP (inactive) with Lte1, a guanine-nucleotide exchange factor located in the bud. This displacement leads to an active form of Tem1 (Tem1-GTP) which activates the mitotic exit network (Bardin *et al.* 2000; Pereira *et al.* 2000). Although the existence of such a checkpoint in animal cells has not yet been characterised, centriole movement has been described in some cell lines including HeLa, L929 and CHO. Piel and co-workers (Piel *et al.* 2001) showed that prior to cell cleavage, the mother centriole moves towards the intracellular bridge connecting the daughter cells. This displacement correlates with a narrowing of this bridge and microtubule depolymerisation in the bridge, suggesting again a link between centrosomes and cytokinesis. Moreover, when the movement of the centriole is impaired cytokinesis is defective (Piel *et al.* 2001).

An alternative role of the centrosome during cytokinesis could be explained by the involvement of γ -tubulin in the process. γ -Tubulin has been located at the minus end

of microtubules forming the midbody and inhibition of γ -tubulin function in mammalian cells or fission yeast led to cytokinesis failure (Julian *et al.* 1993; Shu *et al.* 1995; Hendrickson *et al.* 2001).

Although the exact role of the centrosome in cytokinesis remains unclear it appears to play a crucial role in ensuring the fidelity of this process. The centrosome is also involved in other steps of the cell cycle, as several groups have reported its requirement for cells to progress from G1 to S phase.

F - The centrosome is required for the G1/S transition

When the centrosome is removed from CV-1 or BSC-1 cells during mitosis or S phase respectively, cells keep progressing through the cell cycle and complete mitosis, though a large increase in cytokinesis failure is observed (see above, Hinchcliffe *et al.* 2001; Khodjakov and Rieder 2001). However, the acentrosomal daughter cell arrests in G1 and does not enter S phase. Moreover, when the centrosome is dispersed using antibodies, cells cycle normally, suggesting that the transition from G1 to S requires at least one factor normally associated with the centrosomes and independent of its structural integrity (Bobinnec *et al.* 1998). It is not currently known what centrosomal components are required for the cell to progress from G1 to S. It is also unclear why removing the centrosome stops cell cycle progression. Is there a checkpoint associated with the centrosome or is the centrosome simply required for the process to occur? On

one hand, the checkpoint hypothesis is supported by the existence of the 1182-4 acenrosomal *Drosophila* cell line, which progresses in the cycle normally. On the other hand, the fact that some enzymes and their substrates can be found at the centrosome suggests that it could coordinate molecular events required for the G1-S transition.

G - Regulation of centrosome functions: anchoring of regulatory molecules, the role of AKAPs

The centrosome acts as an anchoring site for molecules regulating cellular functions. A-kinase anchor proteins (AKAPs) target kinases and phosphatases to the centrosome and other cellular locations (see Diviani and Scott 2001 for review). AKAPs have at least two functional domains, one enzyme binding motif and a domain determining the cellular localisation of the proteins. To date three AKAPs have been identified at the centrosome: AKAP350 (also known as AKAP450 or CG-NAP), pericentrin, and AKAP 220 (Diviani *et al.* 2000; Reinton *et al.* 2000). AKAP350 binds Protein kinase A (PKA), PKC ϵ , PKN, protein phosphatase 1, and protein phosphatase 2A (Schmidt *et al.* 1999; Takahashi *et al.* 1999; Takahashi *et al.* 2000). When located at the centrosome these enzymes influence centrosome separation and duplication. The targeting of AKAPs to the centrosome is presumably mediated through a 90 amino-acid sequence, called pericentrin AKAP450 centrosome targeting (PACT) domain, capable of targeting reporter protein to the centrosome (Gillingham and Munro 2000). The anchoring of enzymes to the centrosome through AKAPs is best characterised in the case of pericentrin. It anchors PKA at the centrosome through a unique motif of 100 amino

acids allowing its interaction with the PKA type II regulatory sub-unit (Diviani *et al.* 2000). Pericentrin is targeted to the centrosome in a dynein-dependent manner and it has been shown that dynein is a substrate of PKA (Inaba *et al.* 1998; Young *et al.* 2000). Thus, the PKA associated at the centrosome could be involved in regulating dynein functions.

A possible role for AKAPs would be to bring regulatory molecules close to their substrate to control the specificity of their functions. More generally, AKAPs are thought to integrate several signalling pathways by their ability to interact with proteins involved in signal transduction.

III CENTROSOME CYCLE

Each somatic cell contains a single centrosome that has to replicate once during each cell cycle. This occurs in a semi-conservative manner, ensuring that after mitosis each daughter cell receives a centrosome (Kochanski and Borisy 1990). Centrosome replication must be coordinated with other cellular events. For instance, the centrosome replication must be completed prior to mitosis to ensure equal distribution of the centrosomes to each daughter cell. The cell must control the replication process and stop overproduction of centrosomes when the cell cycle is slowed down to repair DNA damage, for example. Failure to stop the replication process leads to supernumerary centrosomes, to the formation of multipolar spindles and therefore contributes to genetic instability.

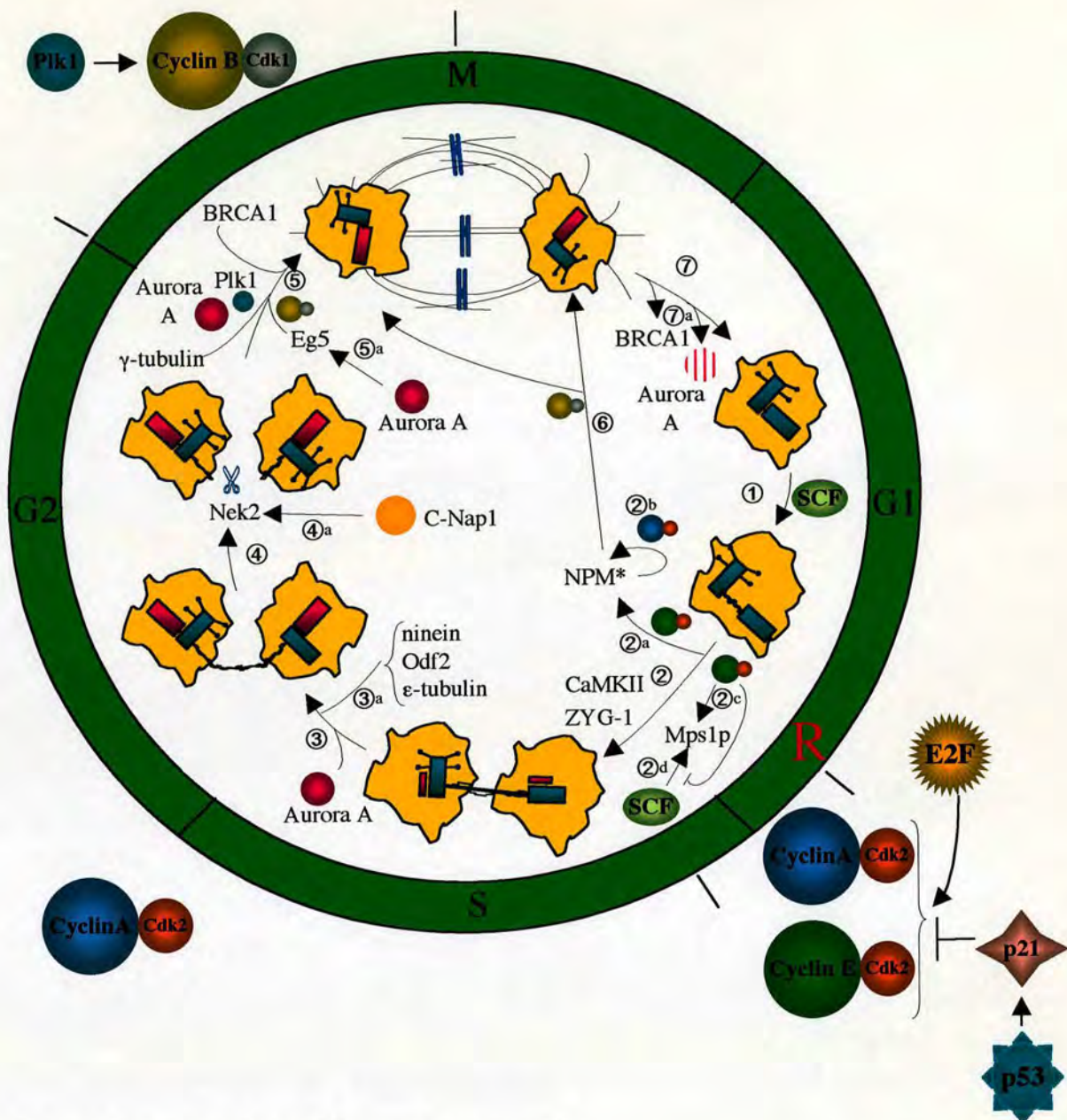


Figure 5: An overview of the centrosome cycle

At the end of G1 phase, the two centrioles lose their orthogonal orientation, maybe through the action of the SCF (Skp1-Cul1-F box) [1]. At the G1-S transition, Cyclin E-Cdk2 phosphorylates nucleophosmin (NPM) [2a]. NPM leaves the centrosome and remains phosphorylated (NPM*), presumably through the action of Cyclin A-Cdk2 [2b], until mitosis. Cyclin E-Cdk2 also phosphorylates Mps1p, preventing its degradation by the SCF [2c, 2d]. These phosphorylation events and CaMKII activity [2] lead to centriole splitting [2]. In *C.elegans* embryos ZYG-1 is involved in this process. Note that Cdk2 activities are under the control of E2F and p53. During S and G2 phases procentrioles form and elongate [3]. Aurora A is recruited to the centrosome in late S phase [3]. In G2 phase, proteins such as ninein, Odf2, and ε-tubulin are recruited to the pericentriolar material of centrosomes with the immature centrioles [3a]. At the end of G2 phase, the two centrosomes separate in a two steps process [4, 5]. First, Nek2 kinase is activated by C-Nap1 [4a] triggering the physical dissociation of the two centrosomes [4]. Second, Aurora A kinase phosphorylates the motor protein Eg5 [5a] which will separate the two centrosomes. Cyclin B-Cdk1 is phosphorylated by Plk1 kinase and is also required for Eg5 activity. In addition γ-tubulin containing complexes are recruited to the centrosome prior the onset of mitosis in a Plk1 and Aurora A dependent manner. NPM is recruited to the centrosome through the action of Cyclin B-Cdk1 [6] and the tumor suppressor protein BRCA1 localises at the centrosome until the end of mitosis [7, 7a]. Aurora A is degraded in the early stages of G1 phase [7a].

A - The centrosome duplication

A somatic cell in G1 contains one centrosome with two centrioles. The first visible event of centrosome duplication is the splitting of the two centrioles which coincides with the loss of orthogonality (also known as disorientation) and occurs in late G1 or early S phase (Figure 5 gives an overview of the centrosome cycle, Kuriyama and Borisy 1981). However, it has been reported that the two centrioles can separate as early as late telophase in some tissue culture cells (Mack and Rattner 1993; Piel *et al.* 2001). Currently, it is not known if the two centrioles are still linked together or if they split before the G1-S transition.

Centriole duplication begins in early S phase by the formation of short daughter centrioles (procentrioles) perpendicular to the mother centriole and slightly separated from the two originals. The newly formed procentrioles will elongate and reach their full length during mitosis or the following G1 phase (Kuriyama and Borisy 1981; Lange *et al.* 2000). Little is known about the molecular events of procentriole formation. New centriole assembly begins with the assembly of an amorphous structure (Figure 6, Dippell 1968; Marshall *et al.* 2001). In *Paramecium tetraurelia* centrin and γ -tubulin concentrate at the assembly site and a pre-pattern for the nine-fold symmetry is set before microtubule assembly (Ruiz *et al.* 1999; Beisson and Wright 2003). In *Paramecium* the elongation process requires γ -tubulin, and in *Tetrahymena* and *Drosophila* γ -tubulin might be required for stabilisation of centriolar microtubules (Ruiz *et al.* 1999; Beisson and Wright 2003). The role of centrin is not fully understood and will be discussed below. Once the precursor is formed microtubules begin to appear, first as a ring of nine single microtubules (formation of tubule A), subsequently converted into doublets (formation of tubule B) and triplets (formation of tubule C). Studies in *Paramecium*

tetraurelia and *Chlamydomonas reinhardtii* demonstrated that the formation of microtubules requires η , ϵ , and δ -tubulin (Figure 6). In *Paramecium sm19* mutants (*sm19* encodes η -tubulin), γ -tubulin mislocalises, indicating that η -tubulin might tether γ -tubulin to the basal body (Ruiz *et al.* 2000). ϵ -Tubulin is required for the cohesion of the centriolar structure in the *Paramecium* basal body (Dupuis-Williams *et al.* 2002; Dutcher *et al.* 2002). In mammalian cells, ϵ -tubulin is a component of the sub-distal appendages and is required for centriole duplication (Chang *et al.* 2003). δ -Tubulin is required for the formation of tubule C of the *Chlamydomonas* basal body (Dutcher and Trabuco 1998; Garreau de Loubresse *et al.* 2001).

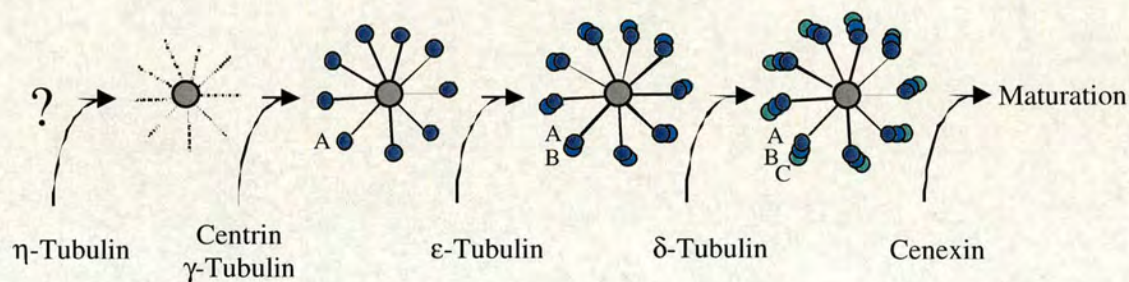


Figure 6: Schematic representation of the centriole assembly pathway.

The initial steps of centriole assembly are unknown (question mark). The required pre-pattern for centriole formation is represented by the nine branched star. Arrows indicate the entry point of the proteins involved in the assembly process and do not represent their time of action. Tubules A, B and C are indicated (adapted from Beisson and Wright 2003).

The two centrosomes, defined as two discrete bodies of PCM, are first visible during S phase, although the exact timing of the transition from one to two centrosomes is unknown. At this stage the two centrosomes are not identical. The one with the mother centriole possesses cenexin/Odf2 and ϵ -tubulin, whereas the other does not (Lange and Gull 1995; Chang and Stearns 2000; Nakagawa *et al.* 2001).

In G2 phase, the immature parental centriole acquires maturation markers such as ϵ -tubulin, ninein and cenexin/Odf2 (Lange and Gull 1995; Chang and Stearns 2000; Piel *et al.* 2000; Nakagawa *et al.* 2001). At about the same time, the amount of γ -tubulin at the centrosome increases three to five fold leading to an increase in microtubule nucleating activity (Khodjakov and Rieder 1999).

In G2/early prophase, the two duplicated centrosomes separate into two distinct MTOCs in a two-step process. First, independently of the microtubules, cohesion between the two centrosomes is disrupted. Throughout the cell cycle, parental centrioles are connected through a proteinaceous structure. The centriole-associated protein, C-Nap1/Cep250 (centrosomal Nek2-associated protein 1), acts as an anchor protein between the proteinaceous structure and the centrioles (Fry *et al.* 1998; Mayor *et al.* 2000). The phosphorylation state of C-Nap1 regulates its association with the centrioles. At the onset of mitosis, the activity of Nek2 (Nima related kinase 2) kinase prevails on phosphatase activity of protein phosphatase 1, and phosphorylation of C-Nap1 leads to the loss of cohesion between the centrosomes. The second step is the physical separation of the two centrosomes. They are separated by the action of plus-end directed motor proteins. One of them, Eg5, is recruited to the centrosome by the dynein/dynactin complex after phosphorylation by Cdk1 (Blangy *et al.* 1995; Sawin and Mitchison 1995; Blangy *et al.* 1997). Each centrosome will be at the centre of the spindle poles and each daughter cell will inherit one centrosome after mitosis. The centrosome duplication is a complex process involving the action of many proteins. It is tightly controlled by regulatory proteins such as protein kinases, calcium binding proteins or E3 ubiquitin ligases.

B - The proteins regulating the centrosome cycle

Centrosome duplication is controlled by the centrosome itself and extrinsic cytoplasmic factors. The intrinsic controls determine the number of daughter centrioles emerging at each cell cycle, whereas the extrinsic ones determine when the duplication starts in relation to nuclear events (i.e. DNA synthesis).

Though centrosome duplication and DNA replication are usually initiated at the same time, several exceptions exist. L929 cells form procentrioles in G1, 4h before the beginning of DNA synthesis (Rattner and Phillips 1973; Hinchcliffe and Sluder 2001). Moreover, mouse embryonic fibroblasts lacking p53 can assemble multiple centrosomes before the onset of S phase (Fukasawa *et al.* 1996). The reasons why some cells initiate and complete centrosome replication in G1 is not understood and is presumably due to differences in regulatory activities. It is also possible that early steps of centrosome replication occur well before DNA synthesis starts. The formation of the two procentrioles could be the physical manifestation of a process started in G1 or even in the previous cell cycle. In S phase, cytoplasmic conditions are permissive for a complete cycle of centrosome reproduction. Artificial prolongation of S phase using hydroxy-urea in CHO cells allows multiple rounds of centrosome duplication without cell cycle progression. The same cells arrested in late G1 with mimosin, or in G2 using a topoisomerase inhibitor, do not overduplicate their centrosome, demonstrating that at these cell cycle stages cytoplasmic conditions are not permissive for centrosome duplication (Balczon *et al.* 1995; Matsumoto *et al.* 1999). Mitosis does not support centrosome duplication but the mother and daughter centrioles can split and separate (Gallant and Nigg 1992).

Thus, temporal regulation of centrosome replication is under the control of cytoplasmic factors at specific cell cycle stages. It has emerged that phosphorylation and proteolysis, two key processes regulating cell cycle progression, are also responsible for the structural and functional transitions occurring during the centrosome cycle.

1) The kinases regulating the centrosome cycle

a) The role of CDK2 in the centrosome cycle

The fact that centrosome replication events are correlated to cell cycle transitions motivated several studies investigating the role of cyclins and cyclin dependent kinases (Cdk) in centrosome duplication. A first link between DNA synthesis and centrosome duplication came from studies demonstrating that Cdk2 is required for both events (Strausfeld *et al.* 1996; Hinchcliffe *et al.* 1999; Lacey *et al.* 1999). The Cdk2-cyclin E complex was identified as the regulator of centrosome duplication in *Xenopus* embryos whereas the Cdk2-cyclin A complex seems to have a predominant role in somatic cells (Hinchcliffe *et al.* 1999; Lacey *et al.* 1999; Meraldi *et al.* 1999). The Cdk2-cyclin E complex phosphorylates nucleophosmin NPM/B23 and stabilises levels of Mps1p kinase, both involved in centrosome duplication (see below, Okuda *et al.* 2000; Fisk and Winey 2001). Cdk2 could have a direct effect on centrosome reproduction by phosphorylating centrosomal components and/or it could act indirectly, affecting pathways influencing centrosome duplication.

NPM/B23 is a substrate of Cdk2

As mentioned above, nucleophosmin NPM/B23 is a substrate of Cdk2. Nucleophosmin was first identified as a nucleolar phosphoprotein with a possible role in ribosome biogenesis (Schmidt-Zachmann *et al.* 1987). Okuda and co-workers identified NPM/B23 as a constituent of the centrosome and a target of Cdk2-cyclin E (Okuda *et al.* 2000). The association of the protein with the centrosome is dynamic. NPM/B23 associates with the unreplicated centrosome and leaves the centrosome at the beginning of the duplication process. At the onset of mitosis the protein is again present at the centrosome (Zatsepina *et al.* 1999; Okuda *et al.* 2000). *In vitro*, the disappearance of NPM/B23 from the centrosome is mediated by the phosphorylation of Thr199 by Cdk2-cyclin E (Okuda *et al.* 2000; Tokuyama *et al.* 2001). What happens *in vivo* is less clear, NPM/B23 could leave the centrosome upon phosphorylation or be degraded by an ubiquitin-dependent proteasome complex present at the centrosome whose activity is required for centrosome duplication (see below). Phosphorylation of NPM/B23 and dissociation from the centrosome are essential for the initiation of centrosome duplication as demonstrated by the use of non-phosphorylatable mutants and antibody micro-injection which both prevent NPM/B23 from leaving the centrosome (or being degraded) and inhibit centrosome replication (Okuda *et al.* 2000). During S and G2 phases the re-association of the protein with the centrosome is prevented, presumably through Cdk2-cyclin A activity. During mitosis the protein re-associates with the centrosome, possibly through the action of Cdk1-cyclin B and each daughter cell receives an NPM/B23-bound centrosome. The role of this protein in the centrosome cycle remains unclear. Nucleophosmin NPM/B23 may be part of the molecular machinery licensing centrosome replication and could act locally to inhibit the duplication process when present at the centrosome.

Cdk2 plays a crucial role in the centrosome cycle at the G1/S transition by licensing centrosome replication, restricting centrosome duplication to once per cell cycle and ensuring the coordination with DNA synthesis. However, Cdk2 is not the only kinase regulating the centrosome cycle. Other kinases involved in this process have been described.

b) MPS1 is required for centrosome duplication

The murine Mps1p (mMps1p, previously known as esk) is an ortholog of the Mps1p kinase of *Saccharomyces cerevisiae*, a protein involved in spindle pole body duplication and the spindle assembly checkpoint. It phosphorylates Spc98p, Spc110p, and Spc42p, a SPB core component (Donaldson and Kilmartin 1996; Pereira *et al.* 1998; Friedman *et al.* 2001; Castillo *et al.* 2002). mMps1p localises to the centrosome throughout the cell cycle (Fisk and Winey 2001). The kinase activity of the protein is required for centrosome duplication since a kinase-dead mutant prevents centrosome replication (Fisk and Winey 2001). Moreover, over-expression of mMps1p in NIH 3T3 cells arrested in S phase triggers centrosome reduplication. Cdk2-cyclin E activity is required for mMps1p-dependent centrosomal duplication and for maintaining the stability of the protein by protecting it from degradation (Fisk and Winey 2001). However, a recent report on the human ortholog, hMps1p, revealed a role in the spindle assembly checkpoint, but no evidence was found for a role in centrosome duplication (Stucke *et al.* 2002). Moreover, the fission yeast ortholog of mMps1p, mph1⁺, is also involved in the spindle checkpoint but not in SPB duplication. Furthermore, the *Caenorhabditis elegans* genome does not encode Mps1p but a unique kinase, encoded by the *zyg-1* gene, involved in centrosome duplication (He *et al.* 1998; O'Connell *et al.* 2001). Hence, the biological

function of Mps1p proteins in higher eukaryotes and their role in the centrosome cycle are still unclear and controversial.

c) ZYG-1, a unique kinase involved in centriole duplication

The *zyg-1* gene of *Caenorhabditis elegans* encodes a protein kinase which does not belong to any known kinase sub-family (Hanks and Hunter 1995). ZYG-1 associates transiently with the centrosome, essentially from anaphase to telophase. *zyg-1* mutants form monopolar spindles with a single centrosome, containing a single centriole. Cell cycle progression is not affected in these mutants, suggesting that ZYG-1 is essential in the initial steps of centriole duplication for daughter centriole formation and seems specifically involved in this process, unlike Cdk2 (O'Connell *et al.* 1998; O'Connell *et al.* 2001). However, no substrates have yet been identified and the exact role of the protein remains unknown.

d) The role of calcium and calmodulin-dependent kinases in the centrosome cycle

Periodic calcium oscillations are observed in *Xenopus laevis* embryos, in particular at the G1/S and G2/M transitions when centrosome duplication and centrosome separation occur respectively, suggesting that calcium may play a role in the centrosome cycle. Matsumoto and Maller found that a transient increase of calcium concentration is required to initiate centrosome duplication in *Xenopus* egg extracts (Matsumoto and

Maller 2002). This effect is mediated through the Calmodulin dependent protein kinase II (CaMKII), a protein previously described at the centrosome (Ohta *et al.* 1990). The mode of action of CaMKII is not clear. Its target(s) are presently unknown, although a number of likely candidates are found at the centrosome, including calmodulin and the centrins (Li *et al.* 1999).

Centrins (centrin 1 to 4) are EF-hand proteins localised at the centrosomes and belong to the calmodulin superfamily of calcium binding proteins. Centrin 2 localises to the centrosome, whereas centrin 3 localises to the PCM (Baron *et al.* 1992; Paoletti *et al.* 1996; Laoukili *et al.* 2000). Depletion of the human centrin 2 in HeLa cells, and over-expression of human centrin 3 in a two-cell stage *Xenopus* embryo lead in both cases to failure of both cytokinesis and centrosome duplication (Middendorp *et al.* 2000; Salisbury *et al.* 2002). In addition, a conditional mutation in *cdc31*, the yeast centrin gene, results in failure of the SPB to duplicate (Baum *et al.* 1986). Finally, phosphorylation of centrin by PKA during G2/prophase correlates with centrosome disjunction, and aberrant centrin phosphorylation was described in breast tumors with amplified centrosomes (Lingle *et al.* 1998; Lutz *et al.* 2001). All these data support a role for centrin 2 and 3 in centrosome duplication. It is, however, not known how these proteins act. In *Spermatozopsis similis*, centrins assemble in a fibrous structure, which emanates from the centriole (McFadden *et al.* 1987). Cdc31p, the yeast centrin protein, localises to a sub-structure of the SPB, called the half-bridge, bridging the original and nascent SPBs. Therefore, centrins could participate in the formation of a structure facilitating subsequent steps of centriole assembly. Alternatively, centrins, as Ca²⁺-binding protein, could act as regulators, modulating the activities of other proteins.

e) Polo and polo-like kinases

Protein kinases from the Polo family have been described in many organisms. Plk1, a mammalian member of this family, regulates many cellular processes such as DNA damage checkpoint activation, regulation of the anaphase promoting complex, and centrosome duplication and maturation (Golsteyn *et al.* 1995; Kotani *et al.* 1998; Smits *et al.* 2000; Golan *et al.* 2002). Plk1 associates with mitotic spindle poles until metaphase at which point it relocates to the midzone.

When Plk1 function is impaired by antibody microinjection, cells display duplicated yet unseparated centrosomes (Lane and Nigg 1996). It has been shown that Plk1 phosphorylates cyclin B at the onset of mitosis therefore activating Cdk1 (Toyoshima-Morimoto *et al.* 2001). HsEg5 is phosphorylated by Cdk1, suggesting that Plk1 regulates HsEg5 activity and therefore centrosome separation (Blangy *et al.* 1995). In *Drosophila Polo* mutants, γ -tubulin does not concentrate on centrosomes, but is scattered throughout the spindle, suggesting that the centrosomal kinase Polo is required for recruiting γ -tubulin to the centrosome (Donaldson *et al.* 2001).

f) Aurora A regulates centrosome replication

Aurora A belongs to a family of serine/threonine kinases. It localises to the centrosome from the end of S phase, after centriole duplication, until early G1 when the protein is degraded (Dutertre *et al.* 2002). Aurora A is not involved in the control of centrosome duplication since overexpression does not lead to centrosome overduplication (Meraldi *et al.* 2002). Inhibition of Aurora A leads to a failure in centrosome separation

both in *Xenopus* and *Drosophila* (Glover *et al.* 1995; Roghi *et al.* 1998). Eg5 has been shown to be phosphorylated by Aurora A presumably in order to trigger centrosome separation. In *Drosophila*, the enzyme phosphorylates the *Drosophila*-transforming acidic coiled-coil (D-TACC) protein allowing the recruitment of this protein to the centrosome (Giet *et al.* 2002). Moreover, in *Drosophila* cells with a delocalised Aurora A, γ -tubulin and centrosomin are not detected at the centrosome, suggesting that Aurora A is required for the recruitment of these proteins at the centrosome (Berdnik and Knoblich 2002).

2) Ubiquitin-mediated proteolysis and regulation of the centrosome cycle

Increasing evidence suggests that ubiquitin-dependent proteolysis plays a role in the centrosome cycle. Skp1-Cul1-F box (SCF) and the anaphase promoting complex/cyclosome (APC/C) are two E3 ubiquitin ligase complexes involved in regulating the centrosome cycle.

a) Role of the SCF E3 ubiquitin ligase in the centrosome cycle

The first evidence for an involvement of SCF in regulating the centrosome cycle came from the observation that Skp1 and Cul1, two core components of the SCF, are localised at the centrosome (Freed *et al.* 1999; Gstaiger *et al.* 1999). Microinjection of antibodies against either of these proteins inhibits centriole separation. Moreover, the use

of a proteasome inhibitor impairs centriole disorientation *in vitro* and prevents centrosome duplication when injected into *Xenopus* embryos (Freed *et al.* 1999). Furthermore, mutations or knock-outs of SCF components in *Drosophila* and mice result in supernumerary centrosomes (Nakayama *et al.* 2000; Wojcik *et al.* 2000). These data suggest a complex role of SCF in the centrosome cycle. It could act in centriole disorientation, which would be a pre-requisite for centrosome duplication, as well as in centrosome separation.

b) The APC/C is involved in centrosome replication events

The anaphase promoting complex/cyclosome has an important role in the metaphase to anaphase transition. It is also involved in the centrosome cycle even though it does not have a centrosomal localisation. It has recently been implicated in controlling the events at the G1/S transition, by preventing the accumulation of cyclin A, which is required to trigger centrosome duplication in somatic cells (Meraldi *et al.* 1999). Recent studies demonstrated that the human Emi1 protein can inhibit the APC/C activity at the G1/S transition, therefore stabilising cyclin A and triggering entry into S phase (Hsu *et al.* 2002). A possible role of the APC/C would be to prevent premature centrosome duplication.

Recently, Nek2 was shown to be a substrate for APC/C ubiquitination through a KEN box motif, involving the APC/C in the control of centrosome separation (Pfleger and Kirschner 2000).

3) E2F is required for centrosome duplication

In somatic cells, centrosome duplication and DNA replication are connected through the common requirement of Cdk2 activity but also through phosphorylation of the retinoblastoma protein and release of E2F transcription factor (Meraldi *et al.* 1999). E2F activates the transcription of *Emi1*, promoting the accumulation of cyclin A (Hsu *et al.* 2002). Interestingly, both cyclin E and A genes are targets of E2F, which might explain the requirement of E2F for centrosome duplication. Alternatively, E2F could promote the transcription of genes encoding proteins involved in centrosome replication.

The centrosome cycle is a mechanism under the control of many regulatory proteins. De-regulation of the cycle leads to supernumerary centrosomes, aneuploidy, genetic instability and cancer.

C - Centrosome and cancer, deregulation of the centrosome cycle

Many reports established a correlation between centrosomal abnormalities in structure and number, aneuploidy and cancer. However, it is not clear if centrosomal abnormalities are the cause or the consequence of tumorigenesis (Lingle and Salisbury 2000). Abnormally duplicated centrosomes are found in tissues or cells carrying mutations in proteins implicated in recognition (BRCA1 and BRCA2) or in response to DNA damage (p53, p21, GADD45a), in protein degradation (Skp2) or in mitosis (Aurora

A). The role of some of these proteins in regulating centrosome duplication begins to be understood.

1) The role of p53 in regulating the centrosome cycle

The *p53* gene is mutated in more than half of cancers and negatively regulates centrosome duplication. p53 is required for the transcription of the Cdk2 inhibitor p21/Waf-1 (el-Deiry *et al.* 1993; Harper *et al.* 1993). Therefore a lack of p53, and p21, leads to an increased Cdk2 activity and overduplication of centrosomes. Loss of p53 also results in an excess of Aurora A leading to cytokinesis failure and polyploidy (Meraldi *et al.* 2002). p53 is present at the centrosome throughout the cell cycle and is thought to regulate centrosome duplication through physical association with the centrosome, in a transcription-independent mechanism (Ciciarello *et al.* 2001; Tarapore *et al.* 2001). The molecular basis of this mechanism is currently unknown. p53 could modulate the activity of centrosomal proteins by direct interaction as previously described for the p53-trk A interaction (Brown *et al.* 2000).

2) BRCA1, a suppressor protein involved in centrosomal functions

Breast cancer suppressor protein 1 (BRCA1) plays an important role in many cellular processes including transcription regulation, DNA damage repair and centrosome

duplication. Mutations in *BRCA1* are associated with the development of familial breast and ovarian cancer. The BRCA1 protein localises to the centrosome during mitosis and binds γ -tubulin *in vitro* when hypophosphorylated (Hsu and White 1998; Xu *et al.* 1999). BRCA1 interacts with many proteins involved in the centrosome cycle including p53 and Cdk2-cyclin A or E, suggesting a complex mechanism of action. When phosphorylated by Cdk2 (or other kinases), BRCA1 fails to interact with the centrosome, whereas the hypophosphorylated form causes centrosome amplification (Hsu *et al.* 2001). Moreover, the absence of *BRCA1* results in centrosome amplification, suggesting that the protein could act as a repressor of centrosome duplication (Xu *et al.* 1999). Since BRCA1 is a regulator of transcription it could also influence centrosome duplication by regulating genes involved in the centrosomal replication process such as *p21* (Hollander *et al.* 1999; Mantel *et al.* 1999).

IV Research subject presentation - aims of thesis

As described above, the G1/S transition is the point where centrosome duplication begins. Moreover, the centrosome is required to progress from G1 to S phases. Although progress has been made in understanding the role of the centrosome in these processes, the molecular events are still not fully understood. It is known that changes occur, both qualitatively and quantitatively, in centrosome composition. As described above, nucleophosmin NPM/B23 is located at the centrosome in G1 phase and leaves it upon

phosphorylation by Cdk2 in S phase. Another example is the phosphorylation of mMps1p at the G1/S transition.

To better understand the mechanism of centrosome duplication and the role of the centrosome at the G1/S transition, we decided to investigate the overall changes occurring in the PCM between G1 and S phase by comparing its composition at these two cell cycle stages.

MATERIALS AND METHODS

All solutions were prepared using de-ionised water (Purite) and where appropriate, were autoclaved before use. Chemicals used were purchased from Sigma or Merck, unless noted otherwise.

I Materials

A - Cell culture

1) Tissue culture cell lines

HeLa JW cells were cultured in Dulbecco's modified Eagle's medium. HeLa S3, Jurkat, K562, HL60 cells, DT40 and DU249 chicken cells were cultured in RPMI 1640. All media were supplemented with 10% foetal calf serum, L-glutamine (2 mM) and antibiotics (penicillin and streptomycin 50 IU each). DT40 medium was also supplemented with 1% chicken serum. These cell lines were grown at 37°C in a 5% CO₂ humid atmosphere.

A6 *Xenopus laevis* cells were cultured at room temperature (22°C) in 65% Leibowitz L-15 media, 35% H₂O supplemented with 10% foetal calf serum, L-glutamine (2 mM) and antibiotics (penicillin and streptomycin 50 IU each).

2) Hybridoma culture

Hybridoma cells were cultured in RPMI 1640 supplemented with 10% foetal calf serum, 10% conditioned H1 (Roche), L-glutamine (2 mM), antibiotics (penicillin and streptomycin 50 IU each), hypoxanthine (100 µM), aminopterin (16 µM) and thymidine (400 nM) at 37°C in a 5% CO₂ humid atmosphere.

B - *Escherichia coli* strains used during this work

E. coli strains were routinely grown at 37°C in Luria Bertani (LB) medium.

XL1-Blue cells were used for cloning and amplifying plasmid DNA. BL21(DE3) cells were used for protein expression.

E. coli genotype:

XL1-Blue: *SupE44 hsd17 recA1 endA1 gyrA46 thi relA1 lac*, F'[*proAB⁺ laqI^r lacZΔM15 Tn10 (tet^r)*]

BL21(DE3): B F⁻ *dcm ompT hsdS (r_B-m_B-)* *galλ*(DE3)

C - Peptides

The peptide CSVTKGVVHQETEIA was synthesised by SIGMA, coupled to keyhole limpet hemocyanin using the N-terminal cystein, and used to immunise two rabbits. The sequence is located in the carboxy-terminal portion of *Xenopus laevis* 4.1R¹³⁵ (x4.1R¹³⁵) (residues 788 to 801).

The peptide EPKELEELREKNESL is located in the tail domain of *Xenopus* NuMA.

D - Primers

Name	Sequence	Comments
XF2	TTCTAGAATGACTACAGAGAAGGGGTTATTA	<i>Xba</i> I cloning site
XF3	TTCTAGATTAAGCTATTTCTGTCTCTTGATG	<i>Xba</i> I cloning site
XF4	TTCTAGAATACACAAAGACTGTAAAAGGTG	<i>Xba</i> I cloning site
XF5	TTCTAGACTACTGGGTGGTGGTGGTACTAC	<i>Xba</i> I cloning site
XF6	GACCCAGAAGCAGACTGTGA	Sequencing primer
XF7	ACTTTATGCGAGGCCTTTGA	Sequencing primer
XF8	CATGGTGTAGACTATGTCAG	Sequencing primer
XF9	GGGAATAGACTCTGTGGATG	Sequencing primer
XF10	GTCGTTTGTCCCATTGCGTG	Sequencing primer
XF11	AAACCGCCGAGCTATTTCTGTCTCTTGATG	<i>Sac</i> II cloning site
HCA66fwd	CGGGGTACCATGGCAGAGATAATTCAGGA	<i>Kpn</i> I cloning site
HCA66rev	CGCGGATCCTAAATGGCCAGTCTGATGCA	<i>Bam</i> H I cloning site
HCA66dir1	CGCGGCCGCCATGGCAGAGATAATTCAGGA	<i>Not</i> I cloning site
HCA66rev1	CCTCGAGTAAATGGCCAGTCTGATGCA	<i>Xho</i> I cloning site
HCA66-1	CGCACTGCGCTTTCATCCAG	Sequencing primer
HCA66-2	AAGTGAGAGGATCATCTGTG	Sequencing primer
HCA66-3	GTCAGAATGCCAATACAAGC	Sequencing primer
HCA66-4	CAATGGCAGACAAACCTGGG	Sequencing primer
MCC-1	CTGCAGTGACCTGAACTCAG	Sequencing primer

MCC-2	TGCTCAATGTGCCTGTCCAC	Sequencing primer
MCC-3	TTCCCATCGCCAAGATTGCT	Sequencing primer
MCC-4	ATTGCTTTTACATTCTCCA	Sequencing primer
MCC-5	GACCAGTCGGGGGATGAAAA	Sequencing primer
MCC-6	CTCCTCCTTCATGGCCATGA	Sequencing primer
MCC-7	GCCCAGGAGCAGGCCTACCT	Sequencing primer

Table 3: Primers used for cloning and sequencing

E - Antibodies

Antibody	Source	Working dilution
Polyclonal anti Septin	Dr. M. Kinoshita, Boston	1/200 IF, 1/300 IB
Polyclonal anti 4.1R	Dr. A. Baines, Canterbury	1/400 IB
Polyclonal anti PCM-1	Dr. A. Merdes, Edinburgh	1µg/ml IF, IB
Polyclonal anti Centrin-3	Dr. M. Bornens, Paris	1/100 IF, 1/200 IB
Monoclonal anti Pericentrin	Dr. A. Dammermann, Edinburgh	1/100 IF
Polyclonal anti γ-Tubulin	Dr. R. Heald, Berkley	1/100 IF
Monoclonal anti γ-Tubulin	Sigma	1/100 IF, IB
Monoclonal anti Lamin B1(clone L-5)	Zymed Laboratories	1/500 IB
Mononoclonal anti Porin	Molecular Probes	1/200 IB
Monoclonal anti α-tubulin (clone DM1A)	Sigma	1/1,000 IF, IB
Polyclonal anti β-tubulin	This study	1/30 IF, 1/50 IB
Polyclonal anti PARP-1	NEB	1/500 IB
Polyclonal anti HCA66	This study	1/100 IF, 1/300 IB
Polyclonal anti MCC1	This study	1/100 IF, 1/300 IB
Texas red and FITC conjugated antibodies for immunofluorescence	Jackson Labs	1/200
HRP-conjugated secondary antibodies for ECL	Amersham	1/10,000

Table 4: Antibodies used during this work

F - Common reagents and buffers

Name	Composition
4x protein sample buffer (Laemli buffer)	250 mM Tris pH 6.8, 9.2% SDS, 0.2% Bromophenol blue , 40% glycerol, 1% 2-mercaptoethanol
6x DNA loading buffer	0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll Type 400 (Pharmacia) in water
Buffer A (for tubulin purification)	50 mM Imidazole/HCl, pH 7.2, 0.5 mM MgCl ₂ , 0.1 mM EGTA, 1 mM 2-mercaptoethanol
BRB80	80 mM PIPES pH 6.8, 1 mM MgCl ₂ , 1 mM EGTA
BRB80-30% glycerol	BRB 80 in 30% glycerol
Cell lysis buffer for centrosome extraction	1 mM Tris pH 8.0, 0.5% Nonidet (NP40), 0.5 mM MgCl ₂ , 0.1% 2-mercaptoethanol, 1 mM PMSF, 10 µg/ml LPC, 10 µg/ml aprotinin
5X column buffer (for tubulin preparation purification)	0.5 M Pipes pH 6.9, 10 mM EGTA, 5 mM MgSO ₄ , 10 mM DTT
Coomassie blue solution	0.1% (w/v) Coomassie blue R250, 10% acetic acid, 50% ethanol, 10% methanol
CSF-XB	XB + 10 mM K-HEPES pH 7.7, 50 mM sucrose, 5 mM EGTA pH 7.7
Energy Mix (EM)	7.5 mM creatine phosphate, 1 mM ATP, 0.1 mM EGTA, 1 mM MgCl ₂
Fixative for aster observation	50% glycerol, 10% formaldehyde (eventually 0.1 µg/ml DAPI) in 1X PBS
Glycerol/PB	80 mM K-Pipes, 5 mM MgCl ₂ , 1 mM EGTA, 1 mM GTP, 33% glycerol (v/v)
GTE	25 mM Tris pH 8.0, 10 mM EDTA, 50 mM Glucose
KPN	50 mM KCl, 50 mM Pipes pH 7.0, 10 mM EGTA, 1.92 mM MgCl ₂ , 1mM DTT, 100 µM PMSF, 20 µM cytochalasin B, 1 µg/ml LPC.
Luria bertani medium (LB)	1% Bactot-tryptone, 0.5% Bacto-yeast-extract, 1% NaCl pH 7.4
Microtubule resuspension buffer (for rhodamine tubulin preparation)	0.1 M Na-Hepes pH 8.6, 1 mM MgCl ₂ , 1 mM EGTA, 40% glycerol
MMR	100 mM NaCl, 2 mM KCl, 1 mM MgCl ₂ , 2 mM CaCl ₂ , 0.1 mM EDTA, 5 mM HEPES pH 7.8
PBS	65 mM Na ₂ PO ₄ , 8.8 mM KH ₂ PO ₄ , 1.37 M NaCl, 2.7 mM KCl pH 7.4
PBS Tween (PBStw)	PBS-0.1% Tween 20
PEM	5 mM PIPES pH 6.8, 1 mM MgSO ₄ , 1 mM EGTA
10X Ponceau S solution	2% (w/v) Ponceau S, 30% (w/v) trichloroacetic acid, 30% (w/v) sulfosalicylic acid

Protease inhibitor cocktail (LPC)	leupeptin, pepstatin, chymostatin 10 mg/ml each in DMSO
Protein electrophoresis buffer	25 mM Tris, 0.192 M Glycine, 0.1% SDS
Separating gel buffer	1.5 M Tris base, 0.4% SDS, pH 8.8
Stacking gel buffer	0.5 M Tris base, 0.4% SDS, pH 6.8
Sucrose gradient buffer	10 mM K-PIPES pH 7.2, 1 mM EDTA, 0.1% 2-mercaptoethanol, 0.1% Triton- X-100
TAE	40 mM Tris-acetate, 1 mM EDTA
TB	10 mM Pipes, 55 mM MnCl ₂ , 15 mM CaCl ₂ , 250 mM KCl, pH 6.7
Transfer buffer	Protein electrophoresis buffer in 20% Methanol.
XB	100 mM KCl, 1 mM MgCl ₂ , 0.1 mM CaCl ₂ , pH 7.8

Table 5: Common reagents and buffers

II CELL BIOLOGY TECHNIQUES

A - Transfection of HeLa JW cells.

Transfections of plasmid DNA in HeLa JW cells were performed by precipitation of DNA with calcium phosphate. Briefly, 60 µl of 2 M CaCl₂ was added to 10 µg DNA, diluted in 420 µl dH₂O with constant vortexing. 480 µl of 2X HEBS buffer (280 mM NaCl, 50 mM HEPES, 1.5 mM Na₂HPO₄, pH 7.15) was then added and the mixture was incubated for 20 minutes at room temperature. The DNA precipitate was then added to 70% confluent HeLa cells for at least 9 hours. Then, the cells were washed with PBS and fresh medium was added.

B - Synchronisation of cells.

1) Arresting Jurkat cells in S phase

Typically, 2×10^9 Jurkat cells were arrested in S phase by inhibition of DNA polymerase δ using a double aphidicolin block. 1 $\mu\text{g/ml}$ of aphidicolin was added to the culture medium for 16 hours. The drug was washed off and the cells were grown for 9 hours under normal conditions then a second block was performed for another 16 hours. The percentage of cells arrested in S phase was determined by FACS analysis (see below).

2) Arresting HeLa S3 cells in prometaphase

HeLa S3 cells were arrested in mitosis by adding nocodazole (200 ng/ml) for 24 hours to the culture media. Cells were then fixed with -20°C cold methanol and their DNA stained with DAPI as described below. Efficiency of mitotic block was monitored by counting mitotic cells.

C - Cell DNA content analysis by flow cytometry

Cells were washed twice in cold PBS then fixed in ethanol 70%, then put at 4°C for at least 30 minutes. Cells were then washed in PBS and incubated for 30 minutes at

37°C in PBS containing 10 µg/ml of RNase A. Propidium iodide (40 µg/ml) was added to the cells which were then analysed by flow cytometry (FACSCalibur Beckton Dickinson). The data were then processed using CellQuest software from Beckton Dickinson.

D - Immunofluorescence

1) Fixation of the sample

Two different methods have been used to fix cells grown on glass coverslips.

Cells grown on glass coverslips were fixed in -20°C methanol for 10 minutes, rehydrated in PBS by three washes of 5 minutes and processed for staining as described below.

Alternatively, cells were fixed in 3.7% formaldehyde-PBS for 10 minutes. Unbound formaldehyde was quenched using 50 mM NH₄Cl in PBS. Cells were then permeabilized in PBS-0.2% Triton X-100 for 5 minutes, washed once with PBS and processed for staining as described below.

2) Staining of the sample

Fixed cells were incubated in PBS-0.1% Tween 20 (v/v) (PBStw) containing 0.5% fish gelatin for 5 minutes, then incubated with the primary antibody for 45 minutes at room temperature in a humidified chamber. After three washes of 3 minutes with PBS, cells were incubated as described previously with a secondary antibody, conjugated to a fluorescent dye (either FITC or Texas Red). Cells were then washed with PBS and the DNA was stained with DAPI (0.6 $\mu\text{g/ml}$) for 3 minutes.

3) Mounting and analysis of the sample

Coverslips were rinsed in PBS and mounted on a drop of antifade mounting medium (Vectashield®, Vector™) placed on a microscope slide.

Slides were examined on a Zeiss Axioskop2 fluorescence microscope. Images were captured using a Zeiss AxioCam camera and AxioVision software and imported into Adobe Photoshop 5.5.

4) Immunofluorescence on suspension cells

Jurkat cells do not attach to glass coverslips. Therefore, to perform immunostaining they were washed twice in PBS by centrifugation and resuspension, then fixed in cold methanol for 10 minutes. Rehydration, blocking and incubation with

the antibodies (primary and secondary) as well as DAPI staining were done as described above. All these steps were done in a microtube. Finally, cells were centrifuged on a glass slide at 200 g for 4 minutes using a Cytospin (Shandon), mounted and analysed as described above.

III BIOCHEMICAL TECHNIQUES

A - Expression and purification of recombinant proteins.

BL21(DE3) bacteria were transformed with the expression vector of interest as described below. Transformed cells were grown from a single colony in LB medium containing the appropriate selection marker at 37°C under shaking until OD₆₀₀ reached 0.6. To induce the expression of the exogenous protein, IPTG (1 mM final) was added to the media, and cells were grown for at least two more hours. Bacteria were pelleted (3,500g, 4°C, 10 minutes) and washed twice in cold PBS containing 0.2 mM PMSF. The pellet was then frozen at -80°C for 16 hours. After thawing, the pellet was resuspended by sonication on ice, in cold PBS containing 0.2 mM PMSF. Cells were centrifuged at 18,000g, 4°C for 15 minutes. The supernatant was stored at -20°C (PBS fraction) and the pellet resuspended by sonication on ice, in cold PBS containing 0.2 mM PMSF and 0.2% Triton X-100. After centrifugation (18,000 g, 4°C, 15 minutes), the pellet was resuspended by sonication and incubated for two hours at room temperature in a buffer

containing 8 M urea, 50 mM Na_2HPO_4 , 0.2 mM PMSF (pH 7.6). The supernatant (Triton fraction) was stored at -20°C . The urea solution was spun at 18,000 g for 20 minutes at room temperature. The supernatant (urea fraction) and the pellet resuspended in dH_2O were stored at -20°C .

An aliquot of each fraction was analysed by SDS-PAGE to determine which of them contained the recombinant protein. The gel was then stained for 1 hour with coomassie solution (0.1% (w/v) Coomassie blue R250, 10% (v/v) acetic acid, 50% (v/v) ethanol, 10% (v/v) methanol). Following a brief rinse in water, the gel was washed in a destaining solution (10% (v/v) methanol, 20% (v/v) acetic acid). When sufficient destaining was obtained, the gel was dried between two cellophane sheets.

B - Purification of GST/His-tagged protein under denaturing conditions

1) His tagged proteins

1 to 2 ml of chelated sepharose slurry (Amersham) was washed twice with dH_2O and incubated for 5 minutes with a 0.1M NiSO_4 solution. The slurry was centrifuged (2 minutes, 200 g) and washed with dH_2O several times. It was then equilibrated with the same buffer in which the protein was kept (in most cases 8 M urea, 50 mM Na_2HPO_4 , pH 7.6) and transferred into a Biorad-Econo column. Binding of the hexa-His-tagged protein to the nickel beads was performed by running the fraction containing the protein through the column. The flow-through was collected and stored at -20°C (flow-through fraction).

The column was washed with 20 ml of a solution containing 8 M urea, 50 mM Na_2HPO_4 , pH 6, and the protein eluted with a solution containing 8 M urea, 50 mM Na_2HPO_4 , pH 4.5.

2) GST tagged proteins

Bacterial pellets were resuspended in PBS containing 1% Triton X-100 and 0.5 mM PMSF, sonicated 3 times for 20 seconds on ice and incubated for 30 minutes at 4°C with gentle agitation. Insoluble material in the lysate was then removed by centrifugation at 80,000 g for 30 minutes at 4°C. The supernatant was then incubated with 1ml washed glutathione sepharose (Amersham Pharmacia) for 30 minutes at 4°C with gentle agitation. Resin was recovered by centrifugation at 500 g for 5 minutes, and washed 5 times with 10 ml PBS before transfer into a chromatography column and elution with 10 mM reduced glutathione in 50 mM Tris pH 8.0 in 15 fractions of 1 ml.

In both cases (His or GST) 10 μl of the fractions were run on a SDS-PAGE and the gel was stained with coomassie solution.

C - Purification of antibodies

1) Affinity purification on a column

Covalent binding of the antigen to the matrix

10 to 20 mg of protein were incubated for 2 hours at room temperature with 500 mg of BrCN-sepharose, pre-washed in 1 mM HCl. The binding efficiency of the protein to the matrix was assayed using the Bradford procedure (Bradford 1976, see below). The free groups remaining on the matrix were blocked using 0.1 M Tris pH 8 overnight at 4°C. The excess ligand was washed with potassium acetate, pH 4.2, then 0.1 M Tris, pH 8.6 three times. The slurry was then poured into a column and washed with PBS.

Purification of the antibody

To bind the antibody to the antigen, 1.5 ml of the serum was run twice through the above column. After two washes with PBS, the antibody was eluted in a solution of high salt/low pH (50 mM glycine, 500 mM NaCl, pH 2.2). 0.5 ml fractions were collected and the pH neutralised using 0.05 volumes of 2 M unbuffered Tris. Protein concentration was assayed according to Bradford. Antibody-containing fractions were pooled together.

2) Affinity purification on a nitrocellulose membrane

The recombinant protein was run on a SDS polyacrylamide gel and transferred onto a nitrocellulose membrane as described. The membrane was then stained with a Ponceau S solution and the area containing the protein cut out, destained and blocked overnight in PBS-0.1% Tween 20 (PBStw) containing 1% fish gelatin. The membrane was then incubated in blocking buffer for 4 hours at 4°C with the primary antibody then washed successively in PBStw containing 1% fish gelatin, PBStw and PBS. The antibody was eluted by incubating the strip in 400 µl of elution buffer (200 mM glycine, 0.5 M NaCl, pH 2.3) for 40 seconds. The acid was then neutralised by addition of 1.2 ml of phosphate buffer (500 mM Na₂HPO₄, pH 9.0). This elution process was repeated twice. Tween 20 and gelatin were added to the eluate (final concentrations 0.1% and 1% respectively) which was then dialysed against PBStw at 4°C for 20 hours.

3) Affinity purification on protein G

In order to purify the monoclonal antibody produced by the hybridoma, 25 ml of filtered tissue culture supernatant were run through a protein G column previously washed in PBS. The column was washed three times with 100 mM Tris pH 8.0 and twice with 10 mM Tris pH 8.0. The antibody was then eluted at low pH in glycine solution as described above. Fractions were collected, pH neutralised and analysed using a 10% SDS-PAGE. Protein concentration was determined by Bradford assay.

D - Protein extraction from eukaryotic cells

Two different methods were used to extract proteins depending of the cell type and the experiment.

1) Standard protein extraction procedure

1x10⁷ adherent cells (HeLa JW, A6) were scraped off a tissue culture dish in 100 µl of lysis buffer (4X sample buffer + 0.1 mM PMSF) and boiled for 10 minutes.

2) Concentrated extract

As an alternative to the above method, in order to prepare very concentrated cell extract, cells (usually HeLa S3) were grown as described and washed twice in 1 volume of cold PBS. They were then washed in 50 ml of cold KPN buffer (50 mM KCl, 50 mM Pipes pH 7.0, 10 mM EGTA, 1.92 mM MgCl₂, 1 mM DTT, 100 µM PMSF, 20 µM cytochalasin B, 10 µg/ml of leupeptin, pepstatin, chymostatin (LPC)) then in 1 ml of the same buffer. After centrifugation at 800 g, the cell pellet was frozen in liquid nitrogen. Cells were lysed by three cycles of thawing-freezing and ground using a mechanical pellet-pestle. The extract was then clarified by ultra-centrifugation at 120,000 g for 45 minutes at 4°C. The soluble fraction was aliquoted and stored at -80°C. The protein concentration was determined using the Bradford procedure and was typically between 30 and 45 µg/µl.

E - Determination of protein concentration

Protein concentration was determined using the Bradford assay (Bradford 1976). Samples were incubated in Protein Assay Reagent (Bio-Rad) diluted 5 fold in dH₂O for ~5 minutes. Absorbance at 595 nm was then determined in a spectrophotometer, and protein concentration determined by comparison to a BSA standard curve obtained under the same conditions.

The protein concentration of cell extracts prepared in sample buffer containing SDS (but not 2-mercaptoethanol or bromophenol blue) was determined by BCA assay (Smith *et al.* 1985). 1ml of working reagent, made up from 100 parts of bicinchoninic acid solution and 2 parts of 4% copper (II) sulphate pentahydrate, was added to 50 μ l of sample and incubated at 60°C for 15 min. Absorbance at 562 nm was then determined, and protein concentration determined using a BSA standard curve.

F - Immunoblotting

Most samples were run on the EC120 Mini Vertical Gel System (E-C Apparatus Co, Holbrook NY). Samples for mass spectroscopy were run on a Protean II xi cell with 1.0 mm spacers (Bio-Rad).

1) Gel electrophoresis (SDS-PAGE)

The separating gel was made of a 30% acrylamide/bisacrylamide stock solution (Perven Biotech) (dilutions to different percentages were used depending on the resolution required), 0.25 volume separating gel buffer (1.5 M Tris base, 0.4% SDS, pH 8.8), 0.1% ammonium persulfate (made freshly every week) and 1/20,000 volume TEMED. The solution was poured between two glass plates, overlaid with water-saturated butanol and allowed to polymerise. The butanol was then discarded and the stacking gel made of 0.25 volume stacking gel buffer (0.5 M Tris base, 0.4% SDS, pH 6.8), 5% acrylamide/bisacrylamide, 0.1% ammonium persulfate and 1/10,000 volume TEMED) poured on top of the resolving gel.

Protein samples were loaded in sample buffer (62.5 mM Tris pH 6.8, 2.3% SDS, 0.05% bromophenol blue, 10% glycerol, 0.25% 2-mercaptoethanol) and run on a SDS-PAGE in protein electrophoresis buffer (MB: 25 mM Tris, 0.192 M glycine, 0.1% SDS). Minigels were run at 25 mA until the dye reached the bottom of the gel. Larger gels were run at 35 mA for 4.5 hours.

2) Protein transfer on nitrocellulose and antibody incubation

Minigels were blotted on a nitrocellulose membrane in transfer buffer (MB containing 20% methanol) at 20 V for 90 minutes. Transfer efficiency was controlled afterwards by staining proteins with a Ponceau S solution. After destaining using PBS,

the membrane was saturated overnight at 4°C with PBStw containing 5% (w/v) non-fat milk powder. The membrane was incubated with the primary antibody for 4 hours at 4°C (or room temperature depending on the antibody), washed 3 times 10 minutes with saturating solution and incubated for 1 hour at room temperature with the secondary antibody conjugated to horseradish peroxidase diluted 1/10,000 in saturating solution.

3) Immunological detection of protein

Using enhanced chemoluminescence

5 ml of 100 mM Tris pH 8.5 containing 25 μ l of 90 mM coumaric acid and 25 μ l of 250 mM luminol were mixed with 5 ml of 100 mM Tris pH 8.5 containing 3 μ l of 30% (w/w) H₂O₂, incubated with the membrane for 1 minute then removed. The nitrocellulose membrane was wrapped in cling-film and placed in an autoradiography cassette to be exposed to an X-ray film for a different lengths of time (usually 10 seconds, 2 minutes and 10 minutes). Films were developed in a Konica SRX-101A developer.

For occasional re-use to allow re-probing, membranes were washed and kept at 4°C in PBS for a few days or stored wet at 4°C wrapped in cling-film.

Quantitative immunoblotting

Quantitative immunoblotting was performed using ECL-Plus reagent as described by the manufacturer (Amersham Pharmacia Biotech). Quantification was performed using a STORM 860 phosphorimager (Molecular dynamics) and ImageQuant v1.1 software (Molecular dynamics).

4) Stripping of nitrocellulose membrane

Prior to incubation with a different antibody, membranes were incubated in stripping buffer (2% SDS, 62.5 mM Tris pH 6.7, 0.1 M 2-mercaptoethanol) for 30 minutes at 60°C with agitation. Blots were then washed twice for 15 minutes with PBStw before blocking them again as described above.

G - Dot Blotting

A nitrocellulose membrane was placed into a blotting chamber. 6 μ g of protein in 50 μ l PBS were put onto the membrane for 30 minutes then the chamber was connected to a vacuum pump and the wells washed twice with PBS. The membrane was removed from the chamber and processed as described above.

H - Preparation of recombinant protein for injection into rabbits.

Several techniques were used to prepare samples

Protein samples were run on SDS-PAGE as described. Gels were rinsed 3 times 10 minutes in dH₂O and stained with aqueous coomassie blue (0.1% Coomassie blue in

25 mM Tris, 0.192 M Glycine) for 1 hour at room temperature while shaking. The gel was destained for 3 times 10 minutes in Tris-glycine buffer (25 mM Tris, 0.192 M Glycine). The desired band was excised with a clean razor blade. The band was cut into smaller pieces, then ground to a fine powder using a ceramic mortar and pestle which had been pre-chilled at -80°C and contained a small amount of liquid nitrogen. Protein powder was stored at -80°C before being sent for injection into rabbits.

Alternatively, when protein samples were pure enough, proteins were dialysed overnight at 4°C against PBS and stored at -80°C before being sent for injection into rabbits.

Rabbits were immunised with 100-200 μg protein in a volume less than 250 μl . Rabbits were injected three times, over a period of 3 months, with the same amount of antigen and a small blood sample was taken from the rabbits for testing one week after injection 2 and 3. Rabbit antibodies were raised either by Eurogentec (Belgium) or by SAPU Diagnostics Scotland (Scotland). Prior to immunisation, pre-immune bleeds from all animals were screened by immunofluorescence staining on cultured cells to avoid use of animals that contained an immune titre against cellular structures.

The mouse immunisation with 4.1C antigen was done by Dr. A. Merdes using 50 μg of protein in 250 μl total volume of PBS. The mouse was injected three times over a period of 1 months with the same amount of protein.

I - Purification of porcine brain tubulin

The purification is based on cycles of polymerisation-depolymerisation of tubulin.

Adapted from Williams and Lee (1982) and Sloboda and Rosenbaum (1982)

Pig brains were carried from the slaughterhouse to the lab in ice-cold buffer A (50 mM Imidazole/HCl, pH 7.2, 0.5 mM MgCl_2 , 0.1 mM EGTA, 1 mM 2-mercaptoethanol). Meninges from 5 brains of freshly slaughtered pigs were removed and brains were chopped into small pieces. Ice-cold buffer A at a ratio of 1 ml/1g of brain was added to brain pieces. Brains were homogenised in the presence of 1 mM PMSF using a blender. The suspension was centrifuged for 25 minutes at 8,000 g at 4°C. The supernatant was mixed with half volume of buffer A, pH 6.8 containing 12 M glycerol then, ATP and GTP were added to the mixture at a final concentration of 1 mM and 0.1 mM respectively (polymerisation step). The mixture was then quickly warmed up to 37°C for 30 minutes and split in 3 different rotors to be centrifuged as follows: 160,000 g for 35 minutes at 37°C in Beckman rotors type 45Ti and 70Ti; 1 hour at 80,000 g in Beckman rotor JA25.50 at 30°C. Pellets were resuspended, using a dounce-homogenizer, in ice-cold buffer A, pH 6.7, 1 mM EGTA. The suspension was incubated on ice for 30 minutes then centrifuged at 4°C, 80,000 g for 30 minutes. A polymerisation step was performed as previously described and microtubules centrifuged for 45 minutes, 37°C at 160,000 g. The pellet was resuspended in ice-cold column buffer (0.1 M K-Pipes, pH 6.8, 2 mM EGTA, 1 mM MgCl_2 , 2 mM 2-mercaptoethanol) aiming for a protein concentration of 35 mg/ml. The mixture was dounce-homogenised as above and the material was frozen in liquid nitrogen and stored at -80°C.

Separation of tubulin from microtubule-associated proteins requires chromatography on a phosphocellulose column.

To prepare the phosphocellulose column (at least a day prior the tubulin prep) 500 ml of ethanol were added to 20 g of dry Whatman p-11 resin, gently stirred, and left to sediment. After removal of the ethanol, the resin was incubated with 500 ml of 0.5 M NaOH for 30 minutes at room temperature, to allow sedimentation. The supernatant was decanted and the resin washed several times with dH₂O until the washing solution reached approximately pH 8. 50 ml of 0.5 M HCl was then added to the resin, gently stirred and sedimented for 30 minutes at room temperature. Supernatant was poured off and replaced with fresh HCl, which was poured off after another 30 minutes. Then, the resin was washed with dH₂O until the washing solution reached pH 5. The resin was then placed in 500 ml of 5X column buffer (0.5 M Pipes pH 6.9, 10 mM EGTA, 5 mM MgSO₄, 10 mM DTT), pH titrated to 6.9 with NaOH and the mixture was left to sediment for few minutes at room temperature before decanting. The resin was washed with 1.5 l of 1X column buffer (0.1 M Pipes pH 6.9, 2 mM EGTA, 1 mM MgSO₄, 2 mM DTT), transferred to a 250 ml cylinder and mixed with buffer. The resin was allowed to settle for few hours, and after decanting of the supernatant, buffer was added to obtain 1.2 times of the slurry volume. The slurry was poured into a 2cm x 45cm column in the cold room. The column was washed at high flow rate (>1.5 ml/minute) with 5 column volumes of 1x column buffer. The last wash before running the column contained 0.1 mM GTP.

Frozen protein was thawed and loaded onto the column. 3 ml fractions were collected and an aliquot was run on a polyacrylamide gel to determine which fractions

contained the pure tubulin. The concentration of protein was determined, as described previously using a Bradford assay. Fractions showing a tubulin concentration above than 1 mg/ml were pooled and $[Mg^{2+}]$ brought to 1mM using $MgSO_4$. Tubulin was flash-frozen in small droplets and stored in liquid nitrogen.

J - Labelling of tubulin with rhodamine

According to Hyman and collaborators (Hyman et al. 1991)

1) Producing polymerisation-competent tubulin

The cycling procedure eliminates tubulin subunits that are not polymerisation-competent and produces a tubulin preparation suitable for in vitro assays.

Fifty milligrams of tubulin were thawed in glycerol/PB buffer (80 mM K-Pipes, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, 33%(v/v) glycerol, pH 6.8). Microtubules were polymerised at 37°C, 30 minutes, then layered onto a cushion of BRB80 buffer (80 mM PIPES pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) containing 60% (v/v) glycerol pre-warmed to 37°C. Microtubules were sedimented for 30 minutes at 190,000 g, 37°C, in a Beckman TLA100 rotor. The supernatant was aspirated, the cushion interface was rinsed twice with dH_2O and the cushion was then aspirated. The microtubule pellet was broken down in ice-cold BRB80 and incubated at 0°C for 15 minutes. The volume of BRB80 was adjusted to yield a protein concentration 10 to 30 mg/ml, assuming half of the initial protein was polymerised. After centrifugation (60,000 g, 15 minutes), the tubulin present

in the supernatant was repolymerised by incubation for 30 minutes at 37°C in the presence of 1 mM GTP and 15% glycerol. Microtubules were sedimented and resuspended in ice-cold BRB80 following the procedure described above. After a last 15 minutes incubation at 0°C, the tubulin in the mixture was sedimented at 60,000 g for 15 minutes at 0°C in a TLA100 rotor. Tubulin present in the supernatant was aliquoted and stored at -80°C or used for labelling.

2) Labelling of tubulin with rhodamine

GTP and glycerol were added to the tubulin in BRB80 (tubulin was now in glycerol/PB: 80 mM K-Pipes, 5 mM MgCl₂, 1 mM EGTA, 1 mM GTP, 33% glycerol (v/v)). Tubulin was allowed to polymerise at 37°C for 30 minutes. Microtubules were layered onto a cushion of 0.1 M Na-Hepes pH 8.6, 1 mM MgCl₂, 1 mM EGTA, 60% glycerol pre-warmed to 37°C, and sedimented in a Beckman SW41 rotor at 200,000 g, 37°C, 50 minutes. The supernatant was aspirated and the cushion washed with resuspension buffer (0.1 M Na-Hepes pH 8.6, 1 mM MgCl₂, 1 mM EGTA, 40% glycerol). Microtubules were resuspended by pipetting and vortexing in a minimal volume of pre-warmed resuspension buffer (100 to 200 µl). 1/10 volume of 100 mM rhodamine (Molecular Probes) in DMSO was added to the microtubules and the mixture was incubated for 10 minutes at 37°C. The reaction was stopped and the pH lowered by addition of 2 volumes of BRB80 containing K-Glutamate and 40% glycerol (v/v). The mixture was loaded onto a 60% glycerol BRB80 cushion and centrifuged for 15 minutes at 60,000 g at 37°C. Prior to resuspension of the pellet in ice-cold BRB80, the

supernatant was removed and the cushion washed as described above. The mixture was incubated at 0°C for 15 minutes then cold-centrifuged for 15 minutes at 60,000 g. Two cycles of polymerisation depolymerisation were performed for the rhodamine-labelled tubulin. The final pellet was resuspended in BRB80, aliquoted, flash-frozen in liquid nitrogen and stored at -80°C.

K - Preparation of cytostatic factor-arrested frog egg extract.

Xenopus laevis egg extracts were prepared according to A. Murray (Murray 1991).

Frogs were primed for ovulation by injection of 50 U of pregnant mare serum gonadotropin (PMSG) on day 1 and 25 U on day 3. Ovulation was induced on day 5 by injecting 150 U of human chorionic gonadotropin (HCG). Eggs were harvested on day 6 by gentle massage of the frogs and collected in MMR buffer (100 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 0.1 mM EDTA, 5 mM HEPES pH 7.8). Eggs were dejellied in an XB solution containing 2% cysteine (100 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, pH 7.8) then washed in CSF-XB buffer (XB + 10 mM K-HEPES pH 7.7, 50 mM sucrose, 5 mM EGTA pH 7.7). After addition of protease inhibitors (LPC: leupeptin, pepstatin, chymostatin 10 µg/ml each) and 10 µg/ml cytochalasin B, the eggs were first compacted in a swinging bucket centrifuge (800 g, 4°C, 1 minute) and after removal of excess buffer, crushed by centrifugation (12,000 g, 4°C, 15 minutes). The cytoplasmic

layer was collected using a needle and syringe via side puncture. The extract was kept on ice to be used immediately or aliquoted and stored at -80°C .

The quality of the extract was assayed by monitoring aster and spindle formation when incubated at room temperature with demembrated frog sperm nuclei. 10 μl of freshly made extract was incubated for various periods of time (typically, 10, 30 and 60 minutes) with sperm nuclei, energy mix (EM: 7.5 mM creatine phosphate, 1 mM ATP, 0.1 mM EGTA, 1 mM MgCl_2) and rhodamine-labelled tubulin. 1 μl of sample was spotted on a microscope slide on top of 4 μl of fixative solution (50% glycerol, 3.7% formaldehyde, 0.1 $\mu\text{g/ml}$ DAPI in 1X PBS). A coverslip was carefully placed on top of the fixed sample allowing visualisation by fluorescence microscopy.

L - Centrosome related techniques

1) Purification of centrosomes

Centrosomes were purified according to Bornens, 1987 (Bornens *et al.* 1987). Briefly, 2×10^9 cells (either Jurkat or DT40), were grown as previously described. 1 h prior to harvesting, the cells were grown in the presence of 0.2 μM nocodazole and 1 $\mu\text{g/ml}$ of cytochalasin D to depolymerise microtubules and actin, respectively. Cells were washed twice with cold PBS and once with cold 0.1X PBS-8% sucrose (w/v). After sedimentation (800 g, 5 minutes, 4°C), they were lysed in 25 ml of lysis buffer (1 mM Tris pH 8.0, 0.5% Nonidet (NP40), 0.5 mM MgCl_2 , 0.1% 2-mercaptoethanol, protease inhibitor cocktail (PMSF 1 mM, 10 $\mu\text{g/ml}$ LPC, 10 $\mu\text{g/ml}$ aprotinin). The

swollen nuclei and cell debris were sedimented at 2,000 g for 15 minutes and the supernatant filtered through a 100 μ m nylon mesh. The lysate was then incubated for 30 minutes at 4°C in the presence of 10 mM K-HEPES pH 7.2, 1 mM EDTA and 600 U of DNase I. The supernatant was overlaid on a discontinuous sucrose gradient (5 ml of 70% sucrose (w/w), 3 ml of 50% sucrose (w/w), 3ml of 40% sucrose (w/w) in 10mM K-PIPES pH 7.2, 1mM EDTA, 0.1% 2-mercaptoethanol, 0.1% Triton- X-100) and centrifuged at 100,000g, 4°C for 1 hour. 0.5 ml fractions were collected from the bottom of the centrifuge tube.

2) Aster formation assay

Centrosome fractions were assayed for their ability to stimulate aster formation both in frog egg extracts and pure tubulin. 1 μ l of each fraction was incubated for 30 minutes either at room temperature with 6 μ l of concentrated mitotic frog egg extract containing energy mix or at 37°C with pure porcine brain tubulin in BRB80-30% glycerol (0.2v) containing 2 mM GTP. In both cases rhodamine-labelled tubulin was added to visualise microtubules. 1 μ l of sample was spotted onto a microscope slide on top of 4 μ l of fixative solution (see above). A coverslip was carefully placed on top of the fixed sample, allowing visualisation by fluorescence microscopy.

3) Stripping of centrosomes

To solubilise the pericentriolar material, purified centrosomes were incubated in 1 M KI (prepared in PEM) at 4°C in the dark. The mixture was centrifuged at 120,000 g for 30 minutes at 4°C. The supernatant, containing the soluble material was then concentrated using a Centricon YM-10 device (cut-off of: 10 kDa; Millipore). The mixture was filtered through the device by centrifugation (5,000 g, 4°C) until almost all the liquid went through the membrane. The retained proteins were then recovered by inversion of the tube and a brief centrifugation at 200 g for 3 minutes. To remove all KI, they were then diluted in PEM, filtered through the device and recovered as described previously. Proteins were boiled for 5 minutes in protein sample buffer and stored at -80°C until loading on a polyacrylamide gel.

4) Immunofluorescence analysis of centrosomal fractions

5 µl of a centrosomal fraction were diluted in 300 µl PEM buffer and loaded in a corex tube onto a PEM-15% sucrose cushion containing a glass coverslip, sitting on an adapter made of araldite®. Centrosomes were sedimented by centrifugation (10,000 g, 4°C, 15 minutes), then coverslips were immersed in methanol at -20°C for 6 minutes. Coverslips were rinsed three times for 3 minutes with PBS and blocked for 3 minutes with PBStw-0.5% fish gelatin. Primary antibody was diluted to a working concentration

in blocking solution and incubated for 30 minutes in a humid chamber at room temperature. The coverslips were rinsed twice in PBStw prior to incubation, as described above, with the secondary antibody diluted 1/100 in blocking solution. Coverslips were rinsed twice in PBS and incubated in 100% ethanol for two minutes. After drying, they were mounted on a drop of mounting medium (Vectashield®, Vector™) placed on a microscope slide. Photometric quantification of immunofluorescence signals were performed from digital image files taken with a 40x/0.75NA lens. Mean pixel values of 13x13 pixels areas were calculated using Adobe Photoshop 5.5 software.

M - Silver staining of polyacrylamide gels

Gels were fixed twice for 15 minutes in a 30% ethanol, 10% acetic acid solution. They were then sensitised for 30 minutes with a fixative solution containing 0.1% $\text{Na}_2\text{S}_2\text{O}_3$ (w/v) titrated to pH 6.0. After three washes of 20 minutes in dH_2O gels were incubated for 30 minutes in a 0.1% silver solution containing 0.01% formaldehyde. Excess of silver was removed by a brief rinse in dH_2O . The gel was developed using a solution of 2.5% Na_2CO_3 containing 0.02% formaldehyde. When sufficient staining was obtained the developing solution was discarded and the development stopped using 1% acetic acid. The bands of interest were cut using a clean razor blade and stored at -80°C .

N - Sample preparation for mass spectrometry analysis

Cut bands were thawed and incubated in 200 mM NH_4HCO_3 , 50% acetonitrile (ACN) at 30°C for 3 times 30 minutes to remove the SDS. Proteins were then reduced by incubation in 20 mM DTT, 200 mM NH_4HCO_3 , 50%ACN at 30°C for 1 hour. The DTT was then removed by three 5 minutes washes in 200 mM NH_4HCO_3 , 50%ACN. Cysteines were then alkylated in 50 mM iodoacetamide, 200 mM NH_4HCO_3 , 50%ACN at room temperature in the dark for 20 minutes. Bands were washed three times in 20 mM NH_4HCO_3 , 50% ACN then cut in small pieces using a clean scalpel, spun 2 minutes at 18,000 g and covered with ACN until they turned white. ACN was decanted and gel pieces were air-dried for 20 minutes. Gel pieces were swollen in 50 mM NH_4HCO_3 containing 2.5 units of recombinant porcine trypsin (Promega). This solution was kept on ice for 30 minutes then transferred to 32°C for 18 hours. After digestion was completed, products were stored at -20°C. Samples were then given to the in-house (ICMB) mass spectroscopy facility for further processing and analysis by MALDI-tof (matrix-assisted laser desorption/ionisation-time of flight) on a Voyager-DE STR-Biospectrometry workstation mass spectrometer (PerSeptine Biosystems).

IV NUCLEIC ACID TECHNIQUES

A - Extraction of plasmid DNA from bacteria

1) from a small volume of culture (mini-prep)

1.5 ml of an overnight grown colony were spun 2 minutes at 10,000 g. The bacterial pellet was resuspended in 200 µl of GTE buffer (25 mM tris pH 8.0, 10 mM EDTA, 50 mM glucose) and the cells were lysed by adding 400 µl of lysis solution (0.2 M NaOH, 1% SDS). The bacterial debris and the chromosomal DNA were precipitated by addition of 300 µl of 3 M sodium acetate pH 5.3 and then pelleted for 15 minutes at 18,000 g. The supernatant was collected and the plasmid DNA precipitated by adding 0.7 volumes of isopropanol. After centrifugation (18,000 g, 15 minutes), the DNA pellet was washed in 70% ethanol and resuspended in 20 µl dH₂O containing 0.5 µg of RNase A. 2 µl was analysed on a 1% agarose gel (usually after a restriction digest).

2) from a large volume of culture

When a larger amount and cleaner DNA was needed (e.g. for transfection experiments) we used the QIAFILTER MIDI-KIT. 100 ml of overnight culture were harvested and plasmid DNA isolated using the QIAfilter Plasmid Midi Kit (QIAGEN)

according to manufacturer's instructions. The yield was determined by measuring absorption at 260 nm.

For multiple plasmid DNA preparations of the same construct, columns were regenerated after use by passing through another volume of elution buffer, followed by one volume of 0.5x TE, 50% ethanol. Columns were stored dry at room temperature.

B - Restriction digest of DNA

Restriction digests were performed for at least 2 hours using enzymes from New England Biolabs. Reactions were performed in the buffer (eventually supplemented with 1 mg/ml BSA) and at the temperature appropriate for the enzyme(s) used (usually 37°C).

C - Agarose gel electrophoresis.

Unless otherwise specified, all agarose gels were made of 1% agarose (Bioline, London) containing 0.3 $\mu\text{g/ml}$ of ethidium bromide in TAE buffer (40 mM Tris-acetate, 1 mM EDTA), and the electrophoresis was performed in the same buffer at 100 V.

D - Purification of DNA from agarose gels

Electrophoresis was performed as described above. After visualisation using U.V. light, relevant bands were cut out of the gel using a clean razor blade and placed into a 1.5 ml tube. DNA was extracted using a Qiagen Gel extraction kit according to manufacturer instructions.

E - Blunting of 5' and 3' DNA overhangs

When possible, restriction enzyme(s) were heat-inactivated and digest reactions were supplemented with 33 μ M of each dNTP and 2.5 U of DNA polymerase I Klenow fragment (New England Biolabs). When it was not possible to inactivate the enzyme(s), DNA was gel-purified and resuspended in restriction digest buffer supplemented with 33 μ M each dNTP and 2.5 U of DNA polymerase I Klenow Fragment (New England Biolabs). Fill-in was performed for 15 minutes at 25°C. Reactions were stopped by addition of EDTA to 10mM and heat-inactivated at 75°C for 20 minutes.

F - Ligation of DNA

Ligations were performed overnight at 16°C using 400 U of T4 DNA Ligase (New England Biolabs) in ligation buffer (50 mM Tris, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA, pH 7.8) at a 3/1 molar ratio of insert/vector. For blunt-end ligations, purified vector was pre-treated with 5 U of alkaline phosphatase for 1 h at 37°C. The reaction was stopped by adding 0.5 mM EDTA and heating at 65°C for 15 minutes. The DNA was purified as previously described.

G - Vector constructions

1) pRSET-MCC₈₂₁ and pRSET-MCC₉₈₇

The full-length cDNA clone of MCC1 (mutated in colorectal cancer 1) was obtained through the IMAGE consortium (IMAGE clone 4111706). DNA of this clone was digested by *Pvu* II and *Nco* I enzymes. The 821 bp and the 987 bp fragments were gel purified and cloned into pRSET-A previously cut by *Pvu* II and *Nco* I or only by *Pvu* II generating pRSET-MCC₈₂₁ and pRSET-MCC₉₈₇ respectively. In the pRSET-MCC₉₈₇ cloning orientation was verified by PCR using T7 and MCC4 primers located in the vector and the insert respectively.

2) Construction of pRSET-HCA66 and pRSET-HCA66₈₇₋₃₆₆

An HCA66 cDNA containing the whole open reading frame was generated by PCR using specific primers (HCA66fwd and HCA66rev; see Table 3), *Taq* DNA polymerase (Roche), and reverse transcription products from randomly primed Jurkat RNAs as a template. The PCR product was gel purified and ligated into pGEM-T[®] vector (Promega) as described. Accuracy of the insert sequence was verified by sequencing. The construct was then digested by *EcoR* I and *Hind* III and cloned into pRSET-C previously cut by the same enzymes. Boundaries were verified by sequencing. pRSET-HCA66₈₇₋₃₆₆ was generated by cutting the full length cDNA (in pGEM-T[®]) by *EcoR* I and *Hind* III, gel purification and cloning into a pRSET-C vector previously cut by the same restriction enzymes.

3) Construction of pCMV-HCA66myc

An HCA66 cDNA containing the whole open reading frame was generated by PCR using specific primers (HCA66dir1 and HCA66rev1; see Table 3), *Taq* DNA polymerase (Roche), and pRSET-HCA66 as template. The PCR product was gel purified and ligated into pGEM-T[®] vector (Promega) as described. Accuracy of the insert sequence was verified by sequencing. The construct was then digested by *Not* I and *Xho* I and cloned into pCMV-Tag5A (Stratagene) previously cut by the same enzymes. Boundaries were verified by sequencing.

4) Construction of pRSET-x4.1R

A x4.1R¹³⁵ cDNA containing the whole open reading frame was generated by PCR using specific primers (XF2-XF3; see Table 3) containing *Xba* I sites, *Pfu* turbo DNA polymerase (Stratagene) and pSP64T-x4.1R as a template (gift from R. Moon). PCR conditions were as described. The PCR products were run on a gel and purified as described. After purification, amplified DNA was cut by *Xba* I, purified again and cloned into pBluescript SK⁺ previously digested by *Xba* I thus generating pBS-x4.1R. The sequence of the insert was verified by sequencing as described.

To generate pRSET-x4.1R, the pRSET-B vector was cut with *Bam*H I and pBS-x4.1R cut by *Xba* I. Both sites were filled-in using Klenow enzyme and ligated as described. Orientation of insertion was verified by PCR using T7 and XF7 primers. Boundaries were verified by sequencing.

5) Construction of GST-x4.1R¹³⁵

To generate GST-x4.1R¹³⁵, the pGEX-4T-3 (Pharmacia) vector was cut by *Eco*R I and pBS-x4.1R cut by *Xba* I. Vector (pGEX-4T-3) and insert (x4.1R) were gel purified, filled-in using Klenow, dephosphorylated and ligated as described. Orientation of insertion was verified by restriction digest. Boundaries were verified by sequencing.

H - Preparation of *E. coli* competent for transformation by heat-shock

E. coli cells (BL21(DE3) or XL1-Blue) were taken from a frozen glycerol stock and grown overnight at 37°C on LB agar plates. A single colony from this plate was inoculated into 2 ml LB and grown overnight at 37°C. 1 ml of this pre-culture was put into 500 ml LB containing 10 mM MgCl₂ and the bacteria were grown with vigorous shaking at 18°C until OD₆₀₀ reached 0.25-0.7. Growth was stopped by placing cells on ice for 10 minutes. They were then harvested at 2,500 g for 10 minutes at 4°C. The pellet was resuspended in 80 ml of TB buffer (10 mM Pipes, 55 mM MnCl₂, 15 mM CaCl₂, 250 mM KCl, pH 6.7) and cells were harvested again. They were then resuspended by swirling in 20ml TB containing 7% DMSO and placed on ice for 10 minutes. Cells were then aliquoted, flash-frozen and stored at -80°C.

I - Transformation of *Escherichia coli* strains.

Supercoiled DNA (5 to 100 ng) or a ligation product was adsorbed on competent bacteria on ice for at least 30 minutes. Cells were heat-shocked at 42°C for 1 minute then incubated on ice for 2 minutes. Cells were grown for 45 minutes at 37°C in LB medium then plated on LB-agar plates containing the appropriate antibiotic.

J - Sequencing

Sequencing was performed using BigDye v3.0 kit (Applied Biosystems).

250 ng DNA were incubated in a final volume of 10 μ l, containing 1.6 pmol of primer and 4 μ l BigDye Terminator v3.0 Ready Reaction mix. The reaction was performed in a Biometra T3 Thermocycler. DNA was first denatured for 2 minutes at 96°C, followed by 25 cycles of 30 seconds denaturation at 96°C, 15 seconds annealing at 50°C, and 4 minutes elongation at 60°C. Reactions were then kept at 4°C.

DNA was then precipitated by adding 50 μ l of solution containing 1.5 μ l of 3 M sodium acetate pH 4.6, 31.25 μ l ethanol and dH₂O. Tubes were then placed at -80°C for 15 minutes before centrifugation at 18,000 g for 30 minutes at 4°C. Pellets were washed with 70% ethanol, centrifuged again for 10 min and dried for 1 hour at room temperature. Samples were then brought to the sequencing facility (ICABP) for loading onto an ABI Prism 377 DNA sequencer (Applied Biosystems).

ABI sequence files were analysed using the Sequencher sequence analysis software (Gene Codes Corporation, Ann Arbor, MI, USA).

K - Polymerase Chain Reactions

PCR was performed in a Biometra T3 Thermocycler

Except when specified all PCR was performed using 2U of *Taq* DNA Polymerase (Roche), 1 mM dNTPs and 25 pmoles of each primer in a PCR buffer (10mM Tris pH 8.3, 1.5 mM MgCl₂, 50 mM KCl). Parameters were: denaturation for 3 minutes at 95°C followed by 30 cycles of 1 minute denaturation at 95°C, annealing 1 minute at T_m-3°C of the lowest melting temperature, polymerisation at 72°C for 1.5 min/kb to amplify. Then, a last polymerisation step was performed at 72°C for 7 minutes. PCR products were kept at 4°C.

L - RNA extraction and RT-PCR.

1) RNA Extraction

RNA was extracted from either HeLa JW or Jurkat cells as described by Chomczynski (Chomczynski and Sacchi 1987). HeLa JW cells were harvested with trypsin then washed with PBS. After centrifugation (800 g, 5 minutes), the cell pellet was resuspended in 1 ml of lysis buffer (4M guanidium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 150 mM 2-mercaptoethanol). 0.1 volume of sodium acetate (2 M, pH 4.0) and 1 volume of water-saturated phenol were added to the lysate and the mixture put on ice for 10 minutes. 0.2 volumes of chloroform were then added and the mixture

was centrifuged for 20 minutes at 4°C (5,000 g). RNAs present in the aqueous phase were precipitated with 1 volume of isopropanol (1 hour, -20°C) and washed with 70% ethanol. The RNA pellet was resuspended in water. The amount of RNA extracted was determined by spectrophotometry at 260 nm.

2) First strand cDNA synthesis

1 μ g of total RNA, 250 ng random primers and 2 mM dNTPs were incubated at 65°C for 5 minutes then quickly chilled on ice for additional 5 minutes. Then, 40 U of RNAsin™ (Promega) and reverse transcription buffer (50 mM Tris pH 8.3, 75 mM KCl, 30 mM MgCl₂, 20 mM DTT) were added and the mixture was incubated for 5 minutes at 25°C. 200 U of SUPERScript™ RNase H⁻ reverse transcriptase (Invitrogen) were added and the tube placed at 42°C for 50 minutes. Heating at 70°C for 15 minutes stopped the reaction. 1/10 of the reverse transcription product was used for the PCR, performed as described above.

RESULTS

Many questions regarding the role of the centrosome at the G1/S transition and the molecular events of centrosome duplication are yet unanswered. Moreover, little is known about the differences in the composition of the centrosome in mitosis compared to its composition in interphase (the recruitment of γ -tubulin at the onset of mitosis is one example). We aimed to investigate these topics by purifying centrosomes to identify centrosomal proteins potentially involved in either of these processes, using mass spectrometry.

We tried to purify centrosomes from different cell lines available in the laboratory. The different attempts made to isolate centrosomes from HeLa JW cells (fibroblast cell line, adherent) were unsuccessful, or too few centrosomes were recovered ($<5 \times 10^6$ centrosomes from 2×10^9 cells). This very low yield was presumably due to the high level of intermediate filaments present in this cell type, therefore impairing the purification of centrosomes. In contrast, the use of Jurkat cells (T lymphoma, growing in suspension), a cell line with few intermediate filaments and a high nucleus/cytoplasm ratio, gave a high yield of purification ($>4 \times 10^8$ centrosomes from 2×10^9 cells, see below). We therefore decided to use this cell line to isolate centrosomes (see below).

I Identification of cell cycle-dependent changes of the PCM

A - Purification of centrosomes from Jurkat cells

Centrosomes were isolated from Jurkat cells using a sucrose step-gradient as described in Materials and Methods (section III-L-1, see Figure 7 for a schematic representation of the isolation procedure). To identify centrosome-containing fractions, an aliquot of each fraction was separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with a γ -tubulin antibody. As shown in Figure 8, γ -tubulin was typically located in fractions 6 to 11, suggesting that centrosomes were present in these fractions. However, the presence of γ -tubulin did not imply that purified centrosomes were functional. Since it was important to determine whether isolated centrosomes remained functional (i.e. to know whether they were able to nucleate microtubules), small aliquots of fractions 6 to 12 were incubated with frog egg extract and tested for their ability to nucleate microtubules and form asters. Asters were typically observed in fractions 7 to 11, 8 to 10 being the fractions where most asters were observed (Figure 7). However, these asters could be formed either by direct nucleation of microtubules from the intact purified centrosome, or by non-functional centrioles that have recruited PCM proteins from the extract, thereby reconstituting a functional centrosome. To discriminate between these two possibilities, fractions 7 to 11 were incubated with porcine brain tubulin below the critical concentration for spontaneous polymerisation of tubulin.

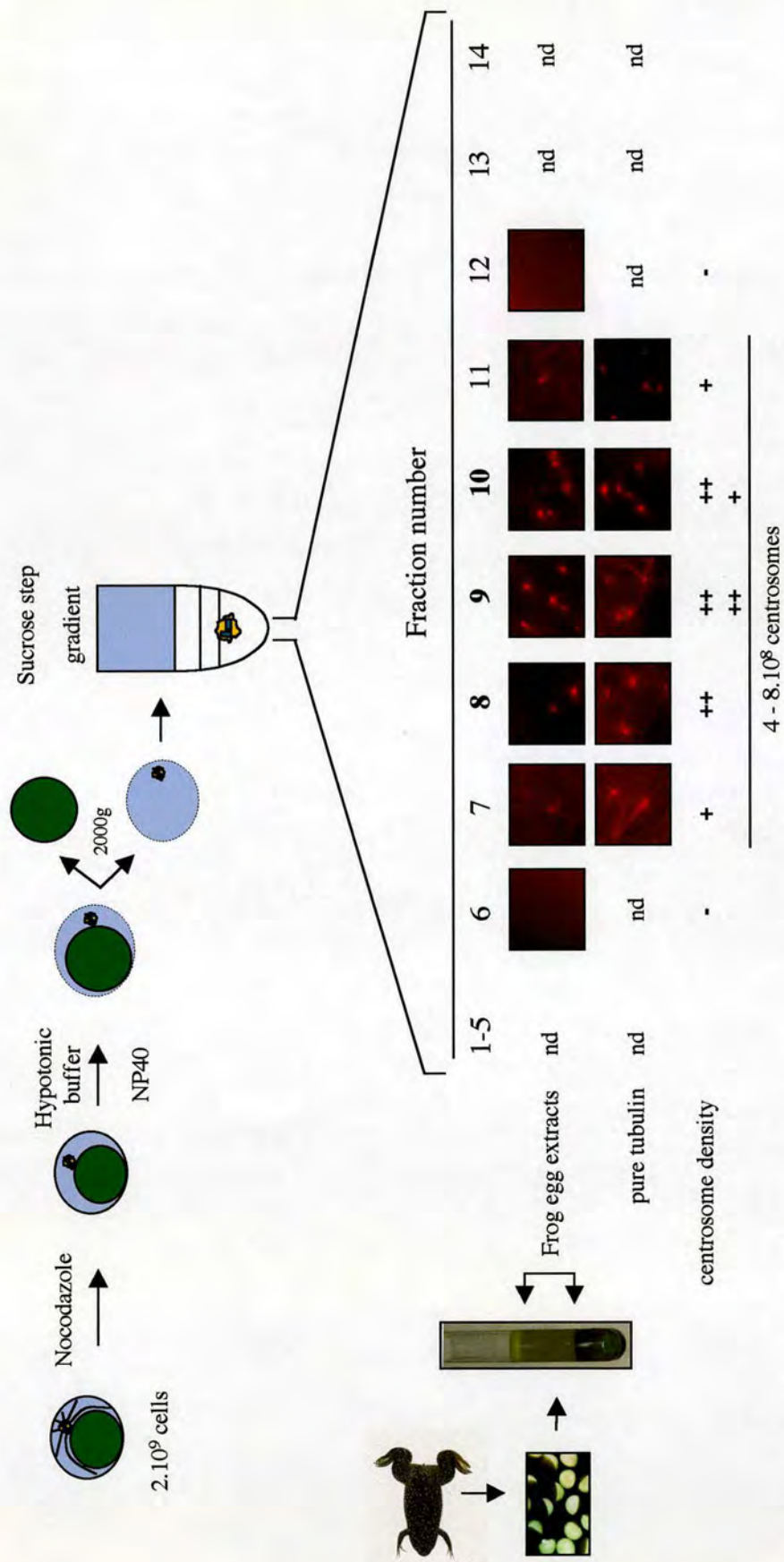


Figure 7: Isolation of centrosomes through a sucrose gradient.

Jurkat cells were treated with nocodazole to depolymerise microtubules, placed in a low salt buffer and lysed using a mild detergent. Swollen nuclei were pelleted and the supernatant loaded onto a sucrose gradient (see Materials and Methods for more details). Fractions collected were incubated with frog egg extracts or pure tubulin (nd: not determined). An evaluation of the number of aster formed is shown (from -: none seen to ++: many). Numbers in bold indicate which fractions were kept for subsequent experiments.

Fractions where most asters were observed (usually 8 to 10) were kept for subsequent experiments.

The number of purified centrosomes per fraction was estimated by extrapolating the number of asters counted in 50 different fields of 0.28 mm² each, observed under a fluorescence microscope. The volume sample in each field was approximately 0.001 µl. From this we calculated that typically, 4 to 8x10⁸ centrosomes were recovered from 2x10⁹ cells, representing ~15% to 30% of the initial number of centrosomes. Approximately 70 to 80% of the isolated centrosomes were located in fractions 8 to 10.

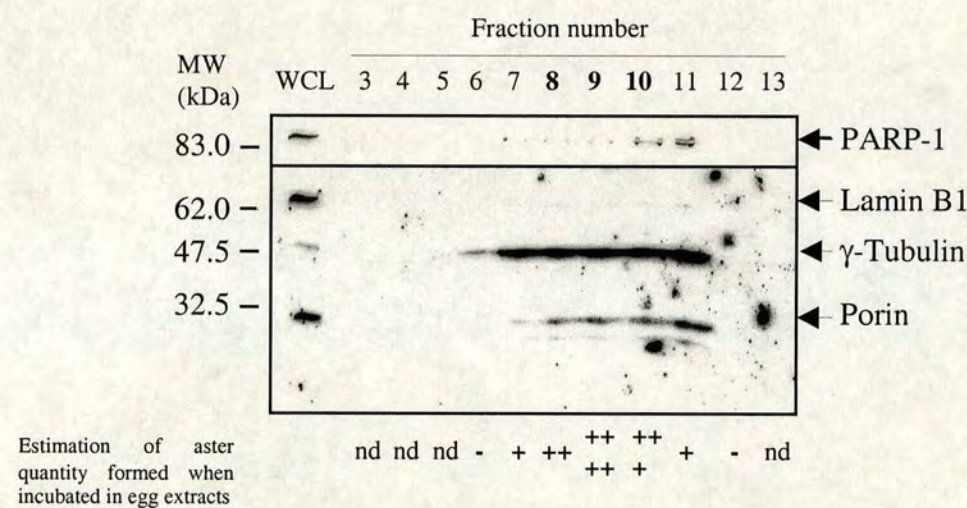


Figure 8: Immunoblotting of sucrose gradient fractions. Jurkat whole cell lysate (WCL) and 10 µl of fractions 3 to 13 from a sucrose gradient were electrophoretically separated on a 10% polyacrylamide gel, blotted onto a nitrocellulose membrane and probed with various antibodies detecting proteins with different intracellular localisation. γ-tubulin monoclonal antibody was used to detect centrosome-containing fractions, whereas porin and lamin B1 monoclonal antibodies were used to detect mitochondria and nuclear lamina-containing fractions respectively. The membrane was then stripped to probe for PolyADP Ribosyl Polymerase (PARP-1) protein. Immunological detection was performed using enhanced chemiluminescence. An estimation of the quantity of asters formed by incubating an aliquot of the fraction in frog egg extracts is shown (from - : none, to ++ : many); nd: not determined.

B - The fractions are highly enriched in centrosomes

We assayed the purity of these fractions by performing several immunoblots, using antibodies against likely contaminants such as mitochondrial or nuclear proteins. The presence of mitochondria was tested using an antibody against porin, a transmembrane protein which forms pores in the outer mitochondrial membrane. A signal was detected in fractions 7 to 11, revealing the presence of mitochondria in the centrosome preparation (Figure 8). We also checked the presence of nuclear contaminants using antibodies directed against the lamina protein, lamin B1, and against the nuclear protein Poly (ADP ribosyl) polymerase 1 (PARP-1). Lamin B1 and PARP-1 signals were detected in fractions 7 to 11, suggesting the presence of nuclear protein contaminants in the centrosome fractions (Figure 8). However, when comparing the γ -tubulin signal with porin, lamin B1, and PARP-1 signals in the whole cell lysate and the centrosome containing fractions, it appeared that γ -tubulin was highly enriched in these fractions. These results demonstrated that fractions 7 to 11 were highly enriched in centrosomes but not in mitochondria or nuclear components.

C - Isolation of "G1" and "S" centrosomes

In order to compare the protein composition of centrosomes from cells in G1 with those from cells in S phase, centrosomes were isolated from unsynchronised cells and cells arrested in S phase.

Three different methods were tested to arrest the cells in S phase: double thymidine block, hydroxyurea or double aphidicholin block. Time course and dose-effect experiments demonstrated that the double aphidicholin block gave the highest number of cells arrested in S phase (~80% versus ~65% for thymidine block and ~60% for hydroxyurea). This method was therefore chosen to arrest the cells in S phase.

1) A Jurkat cell population enriched in S phase

Jurkat cells were blocked in the early stages of S phase using aphidicolin, which inhibits DNA polymerase δ (see Materials and Methods). The blocking efficiency and cell cycle profile were determined using flow cytometry analysis (Figure 9A). Typically, ~80% of cells were arrested in S phase after treatment compared to ~25% in an untreated culture (Figure 9A). Treatment with aphidicolin diminished the proportion of cells in G1 (~9% in the aphidicholin-treated population compared to ~65% in the untreated cells) and decreased the proportion of cells in G2/M (~4% of the aphidicholin-treated cells compared to ~10% of the untreated cells). However, the number of apoptotic cells slightly increased (~9% of the aphidicholin-treated cells compared to ~4% of the untreated cells). Thus, the aphidicolin treatment diminished by more than 7 times the number of cells in G1 and increased, more than 3 times, the number of cells in S phase, allowing us to compare the unsynchronised population with the S phase-arrested population.

2) Centrosomes do not over-duplicate upon treatment of Jurkat cells with aphidicolin

As described in the introduction, a prolonged arrest in S phase can trigger over-duplication of the centrosome in some cell types. To test if the double aphidicolin block led to supernumerary centrosomes, aphidicolin-treated cells were stained for γ -tubulin. Figure 9B shows a representative example of aphidicolin-treated Jurkat cells stained with an anti- γ -tubulin antibody. We did not detect an increase in the number of cells with more than two γ -tubulin foci when compared to the unsynchronised population, suggesting that Jurkat cells do not support centrosome over-duplication in these conditions. Therefore, centrosomes isolated from aphidicolin treated cells will not have gone through several rounds of duplication and will be in their "normal" state.

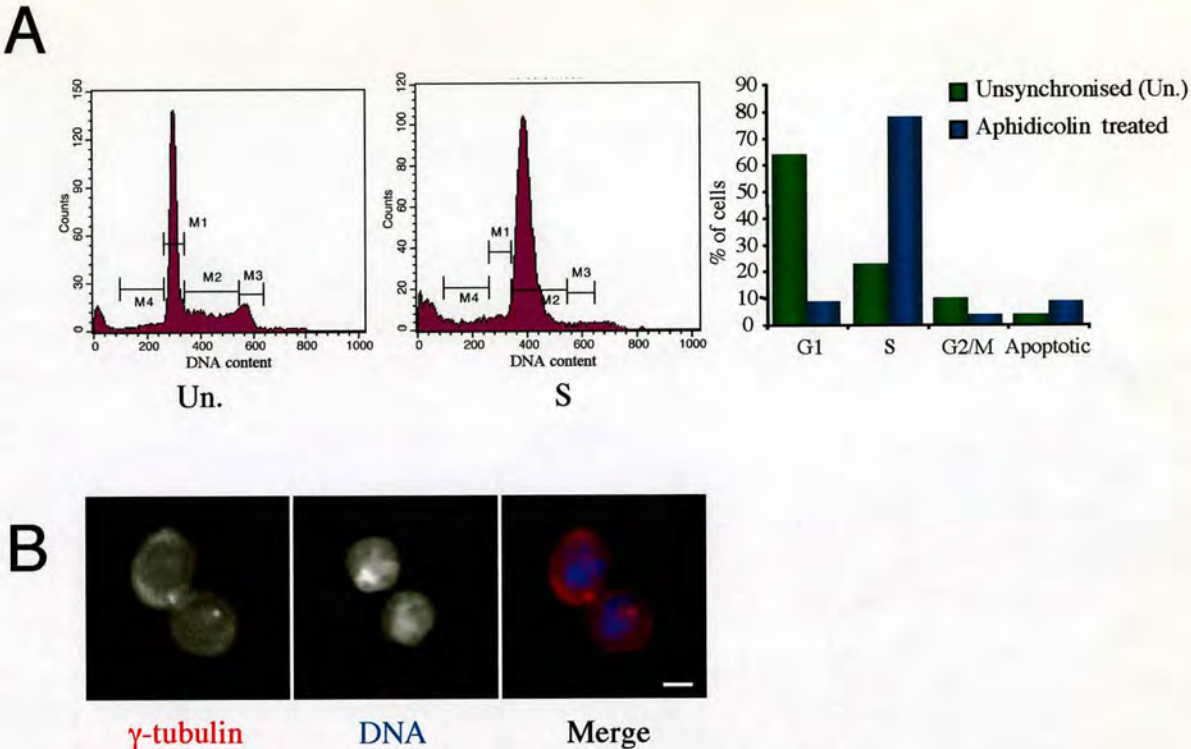


Figure 9: Jurkat cells arrest in S phase when treated with aphidicolin.

Jurkat cells were treated with aphidicolin as described in Materials and Methods. **A**, left panel, DNA content determined by FACS analysis on untreated (Un.) and aphidicolin-treated (S) Jurkat cells. Segments M1, M2, M3 and M4 delimit the area of the graph used to determine the number of cells in different cell cycle stages (M1: G1 phase cells, DNA content: 2n; M2: S phase cells, DNA content: >2n, <4n; M3: G2/M phase cells, DNA content: 4n; M4 apoptotic cells, DNA content <2n). The right panel shows a quantification of the FACS results: percentage of G1, S, G2/M or apoptotic cells was determined by integration of the signal (count) in segments M1, M2, M3 and M4 respectively. **B**, Immunofluorescence on aphidicolin treated cells: cells were stained for γ-tubulin (red); DNA was stained with DAPI (blue). Bar 5 μm.

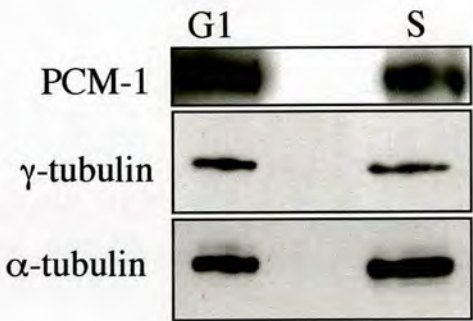


Figure 10: Immunoblot of various centrosomal proteins on isolated centrosomes.

“G1” (G1) and “S” (S) centrosomes were electrophoretically separated and blotted onto a nitrocellulose membrane which was probed with PCM-1, γ-tubulin and α-tubulin antibodies. Immunological detection was performed using ECL-Plus reagent.

3) Quantitative analysis of the presence of some known proteins at "G1" and "S" centrosomes

We isolated centrosomes from an unsynchronised culture of Jurkat cells, therefore mainly in G1, and from a population of cells arrested in S phase. To simplify, these centrosomes will be referred as "G1" centrosomes and "S" centrosomes respectively. We first tested whether there was a quantitative difference in the presence of known centrosomal proteins in "G1" and "S" centrosomes. Fractions 8 to 10 were pooled, an aliquot was subjected to SDS-PAGE and transferred onto a nitrocellulose membrane, which was probed with various available centrosomal antibodies. We did not detect any significant differences in the amount of PCM-1 and γ -tubulin present at the centrosome from unsynchronised or S phase cells (Figure 10). However, the detection method did not allow an accurate measurement of protein amount, and subtle changes could be missed. We therefore decided to compare the protein amount on "G1" and "S" centrosomes using immunofluorescence. Centrosomes were centrifuged onto coverslips, fixed and immunostained for various centrosomal proteins as described in Materials and Methods (Figure 11A). As expected, intensity of α -tubulin signal was identical in both centrosome populations. While performing the immunostaining experiments on centrosomes, we noticed that fractions from aphidicolin-treated cells contained cellular debris, which were stained by the antibodies, whereas centrosomes from untreated cells did not. No differences were observed in the signal intensity of γ -tubulin from "G1" or "S" centrosomes, confirming the results obtained by immunoblotting (Figure 11, Table 6). We also tested the amount of centrin and pericentrin present in those centrosomes (Figure 11, Table 6). In both cases, the amount of proteins detected did not change significantly.

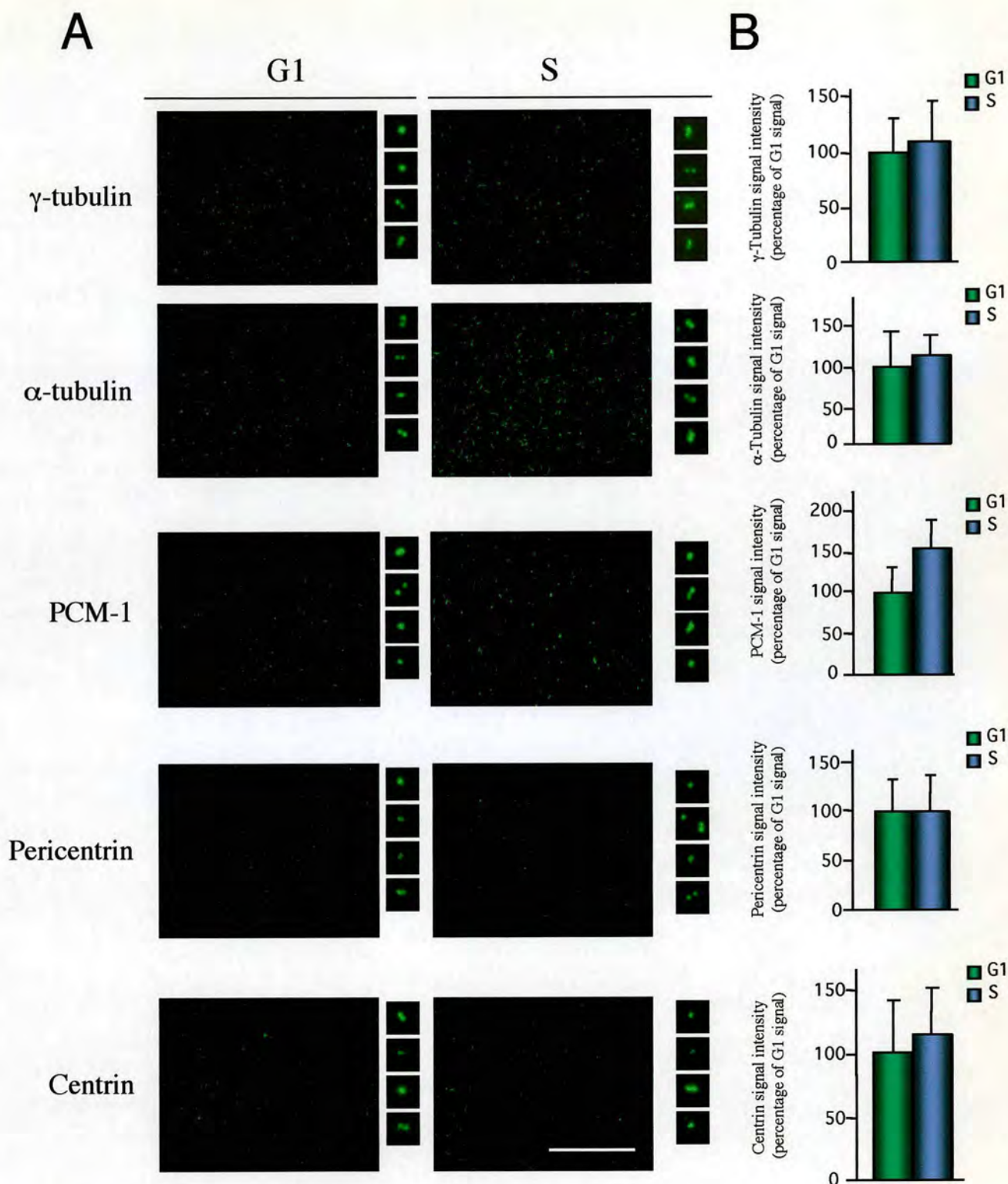


Figure 11: Immunofluorescence of centrosomal proteins.

A, Centrosomes purified from a culture mainly in G1 (G1) or synchronised in S phase (S) of the cell cycle were spun down on coverslips and stained for several known centrosomal proteins. On the right hand side of each panel are four magnified areas showing centrosomes representative of the field. Bar 20 μ m. **B**, Quantification of the signal observed in **A**. Values are expressed as arbitrary units (a. unit) and should not be compared between different proteins. Photometric quantification of immunofluorescence signals were performed from digital image files taken with a 40x/0.75NA lens. Mean pixel values of 13x13 pixels areas were calculated using Adobe Photoshop 5.5 software.

Interestingly, PCM-1 signal in "S" centrosomes was ~150% the signal observed in "G1" centrosomes, suggesting that PCM-1 is recruited at the centrosome in S phase (Figure 11 and Table 6).

	Signal intensity in G1 (a.u.)	Signal intensity in S (% of G1 signal)
α -tubulin	100 \pm 48.10	114.59 \pm 26.59
γ -tubulin	100 \pm 31.12	110.70 \pm 36.66
PCM-1	100 \pm 30.19	152.85 \pm 35.02
centrin	100 \pm 41.40	114.81 \pm 36.58
pericentrin	100 \pm 33.09	100.08 \pm 36.24

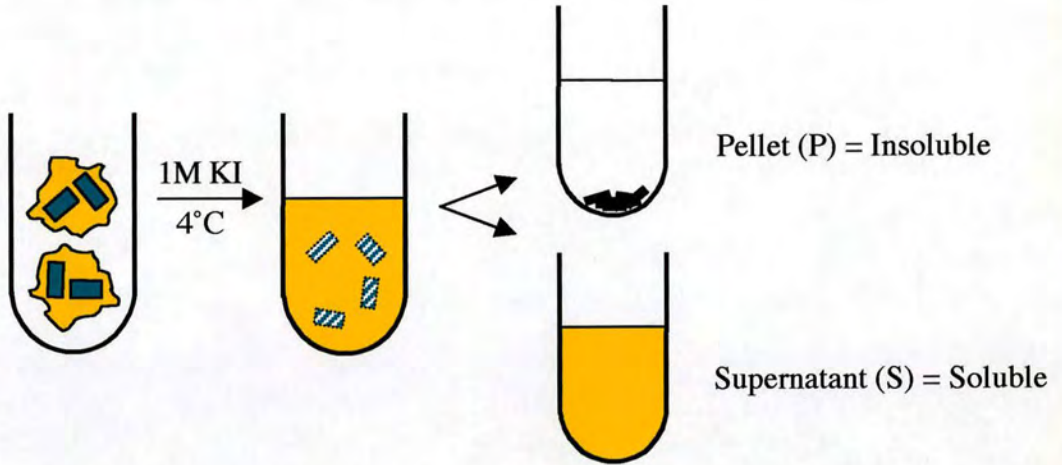
Table 6: Quantification of the signal of various centrosomal proteins on "G1" centrosomes and "S" centrosomes. Signal intensity in "G1" centrosomes was set to 100 (a.u.: arbitrary units). Signal intensity in "S" centrosomes is expressed as a percentage of the corresponding G1 signal. Values should not be compared between different proteins.

4) Stripped centrosomes do not nucleate tubulin

We were interested in identifying changes occurring in the pericentriolar material of "G1" and "S" centrosomes. Treatment of centrosome fractions with potassium iodide (KI), a chaotropic salt, was described to dissociate the centromatrix and the PCM (Schnackenberg and Palazzo 1999). Stripping the centrosomes would also have the advantage to decrease the system complexity and then facilitate the analysis. In our hand, the soluble fraction represented approximately 40-50% of the total material as determined by protein quantification using a Bradford assay. This ratio is lower than the one obtained by Schackenberg and collaborator in *Spisula solidissima* centrosomes (Schnackenberg *et al.* 1998). The difference might result from differences in the composition or architecture of *Spisula solidissima* and human centrosomes.

A time course of KI incubation was performed in order to determine how long the centrosomes should be treated to dissociate the soluble from the insoluble fraction (Figure 12). An aliquot of both fractions was separated by SDS-PAGE, then probed for γ -tubulin, a PCM component. The γ -Tubulin signal in the insoluble fraction decreased gradually with increased time of treatment to become barely detectable after one hour, whereas the signal increased in the soluble fraction, indicating that the PCM was gradually removed from the centromatrix (Figure 12B). The stripping efficiency was estimated to be 95% after 1 hour of treatment. When these stripped centrosomes were incubated with pure tubulin no asters were observed (Figure 13), confirming the stripping efficiency. When incubated in frog egg extracts, stripped centrosomes nucleated tubulin, and asters were observed. Thus, stripping with KI removes the ability of the microtubules to nucleate tubulin. This ability was restored when centrosomes were incubated in egg extracts from which they recruited PCM components (Figure 13).

A



B

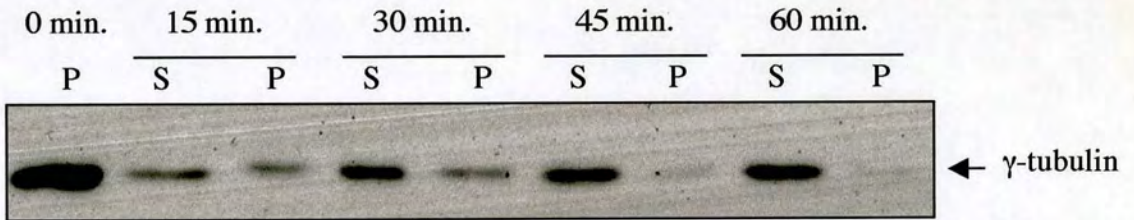


Figure 12: Stripping of centrosomes with KI.

A, Schematic representation of centrosome stripping. Centrosome fractions were incubated with 1M KI at 4°C in the dark and the soluble fraction was separated from the insoluble fraction by ultracentrifugation. **B**, Time course of centrosomes incubated with 1M KI. Fractions (insoluble pellet: P, Soluble: S) were immunoblotted with an anti γ -tubulin antibody. Immunological detection was performed using enhanced chemiluminescence. Time indicates duration of KI treatment.

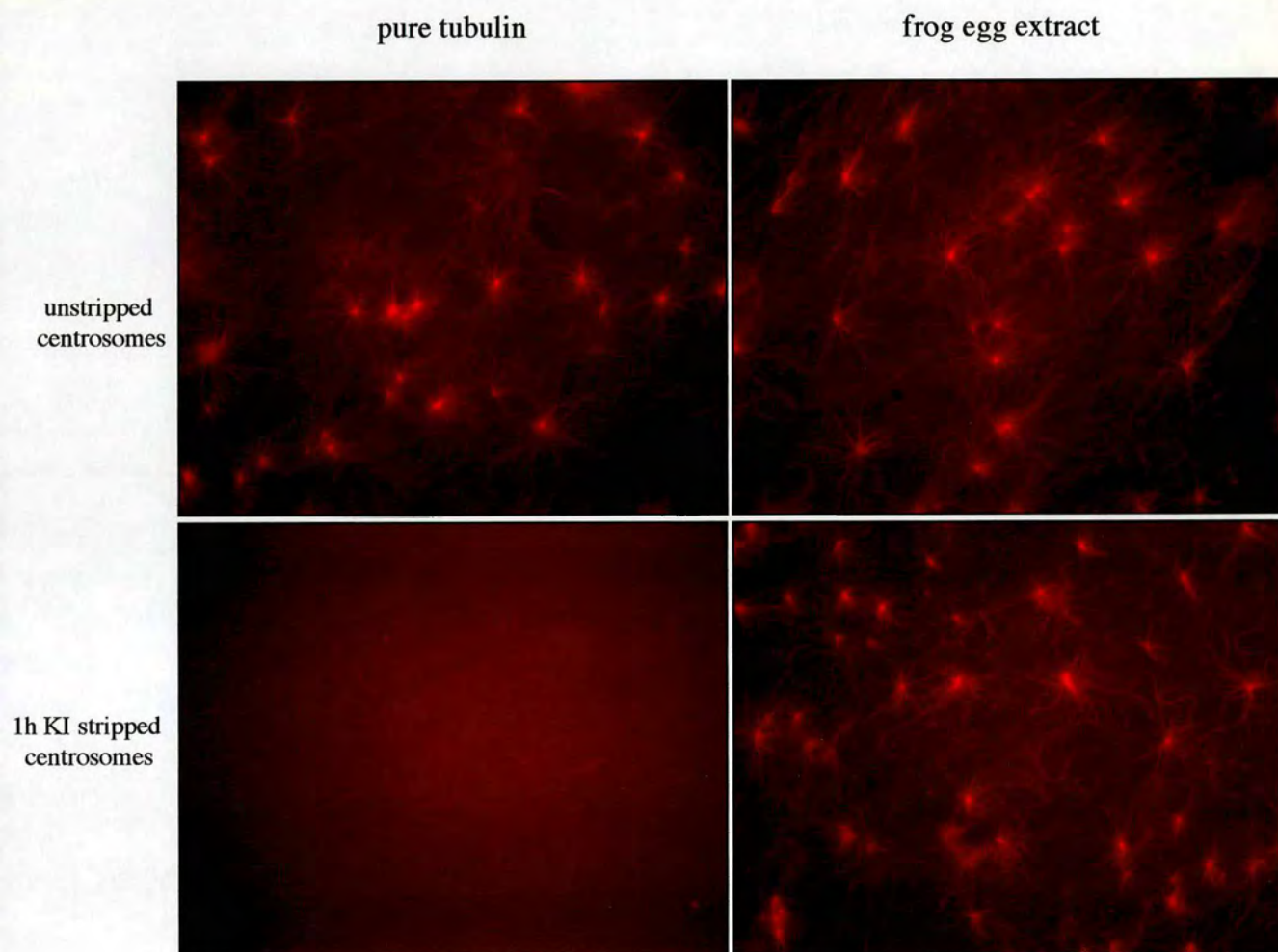


Figure 13: Stripped centrosomes do not form asters in pure tubulin, but can be reconstituted in frog egg extracts.

Centrosomes or KI-stripped centrosomes were spun down on coverslips and incubated either with pure tubulin or with either frog egg extract, then incubated to allow aster formation as described in Materials and Methods. To visualise microtubules, assays were supplemented with rhodamine-labelled tubulin.

D - There are differences in the PCM composition of "G1" and "S" centrosomes

Centrosomes were purified from unsynchronised and S phase-arrested cells and treated for 1h with 1M KI to separate the soluble and insoluble fractions. Before loading onto a gel, the soluble fraction was concentrated and the KI removed using a centricon device as described in Materials and Methods. Centrosome preparations were run in parallel on 6.8%, 9% and 12.5% gels to obtain maximum resolution of high and low molecular weight proteins (Figure 14). By comparing the PCM from two different "G1" and "S" centrosome preparations on each gel, several differences in protein amount of silver stained bands were noticed. Two bands (n° 2 and 5) showed a greater protein amount in the PCM from "G1" centrosomes when compared with the PCM from "S" centrosomes. On the other hand, nine bands (n° 1, 3, 4, 6-11) showed a greater amount of protein in the PCM from "S" centrosomes. All these bands were cut out of the gel and analysed by MALDI-tof (matrix-assisted laser desorption/ionisation-time of flight) mass spectrometry. Because one band may contain more than one protein it was sometimes not possible to identify unambiguously these proteins. This was the case for proteins of bands n° 1, 3, 4, 8, 9 and 11. Results are summarised in Table 7.

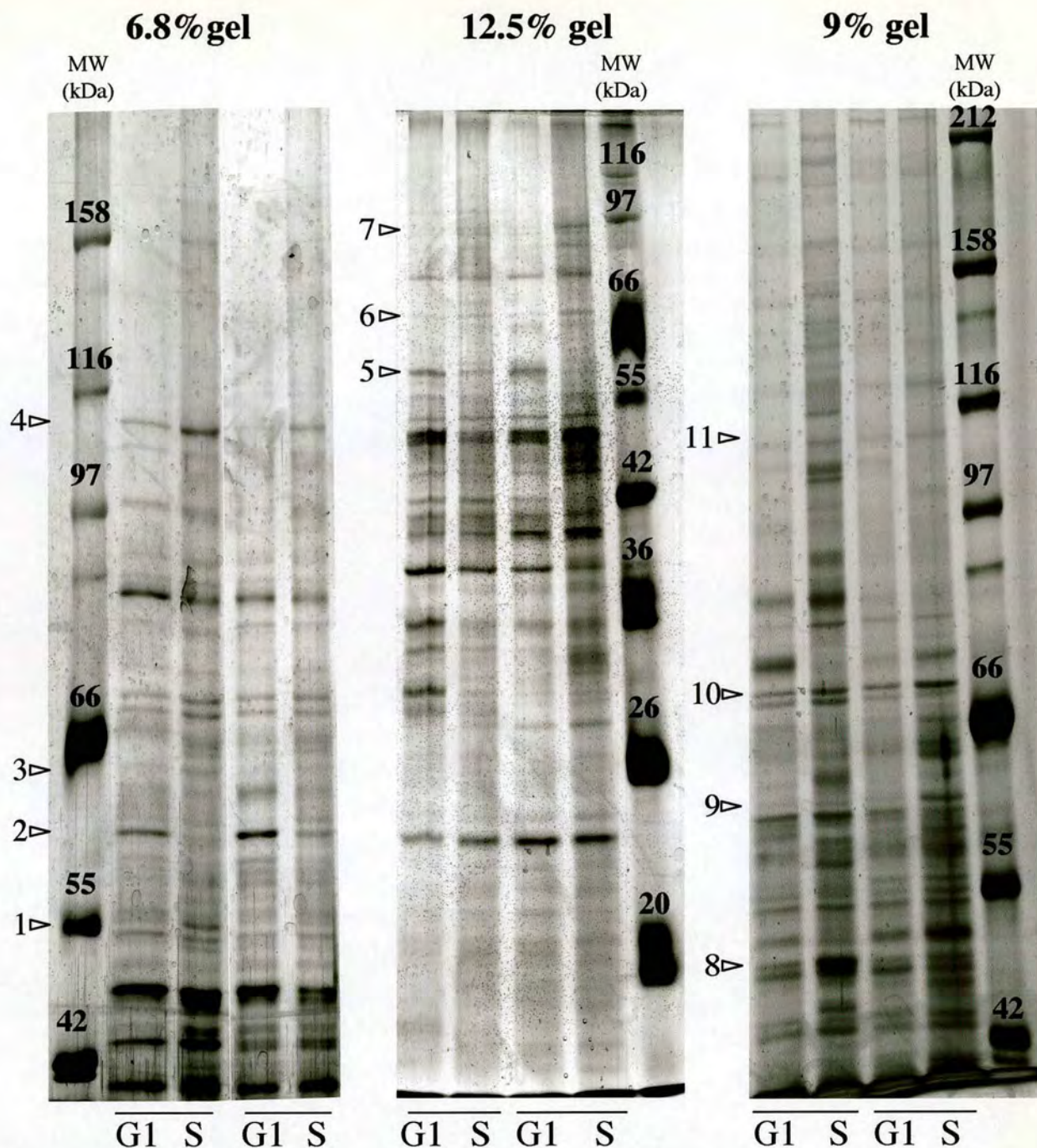


Figure 14: There are differences in the soluble fraction of “G1” and “S” centrosomes. Independent “G1” (G1) and “S” (S) centrosome preparations were incubated in 1M KI, for 1hour in the dark at 4°C, and the soluble fractions separated on 6.8%, 12.5% and 9% gels. Gels were silver stained, and bands enriched in either fractions (G1 or S) were cut out and processed for MALDI-tof mass spectrometry. Numbers 1 to 11 represent the different bands which were cut out of the gels.

Among the 8 proteins identified, only hsp73 (bands n° 6 and 10) was previously described as a component of the centrosome and shown to colocalise with pericentrin, although it is not a *bona fide* centrosomal protein (Brown *et al.* 1996a; Brown *et al.* 1996b). Eukaryotic translation initiation factor 4 α (band n° 8) is a very abundant protein in the cell and is likely to come from ribosomal contaminants of the preparation. The Epstein-Barr nuclear antigen 2 (EBNA-2) and the putative protein MGC:790 (band n° 11) are presumably nuclear contaminants. Pyruvate kinase (band n° 2 and 5) is a cytoplasmic enzyme involved in the metabolism of phosphoenolpyruvate to pyruvate (the last step of glycolysis). The identity of the proteins present in band n° 3 is unclear: aminopeptidase is a cytoplasmic protease, and hnRNP L a nuclear protein which is probably also a nuclear contaminant.

Septins (found in band n° 1) are guanine nucleotide binding proteins required for cytokinesis (see Kinoshita and Noda 2001 for review). As described in the introduction, the centrosome has been implicated in this process, therefore the presence of septin at the centrosome could explain its requirement for cytokinesis. Thus, we chose to investigate whether septin was a centrosomal protein and study its function at the centrosome.

The identified proteins might have a role in regulating centrosome duplication and/or cell cycle progression. Among these proteins, two of them, HCA66 (band n° 9) and MCC1 (band n° 4 and 7), were linked to cancers (Kinzler *et al.* 1991; Wang *et al.* 2002). Since defects of centrosome duplication can lead to supernumerary centrosomes and ultimately to cancer, HCA66 and MCC1 could be implicated in centrosome duplication events. We therefore decided to focus our attention on these two proteins in order to investigate their role in centrosomal events as well as in cell cycle progression.

	\pm in S	Name	MW (kDa)	MWcut (kDa)	Mowse Score	Coverage	Comments
1	+	Septin	49	55	1.6×10^4	n/a	Role in cytokinesis.
		Glutamate dehydrogenase	62		1.1×10^5	n/a	
2	-	Pyruvate Kinase	58	60	2.1×10^9	28%	
3	+	Aminopeptidase	52	62	6.7×10^4	43%	Nuclear protein
		hnRNP L	64		2.3×10^8	19%	
4	+	MCC1 BC007788	100 72	105	4.1×10^6 1.8×10^4	18% 21%	Mutated in colorectal cancer. Eukaryotic translation initiation factor 4 γ like
5	-	Pyruvate Kinase	58	60	5.2×10^{11}	n/a	
6	+	Hsp73	73	70	4×10^5	15%	Described at the centrosome.
7	+	MCC1	100	95	1.5×10^6	20%	see 4
8	+	Eukaryotic translation initiation factor 4 α	50	50	1×10^4	21%	
9	+	HCA66	66	60	2.3×10^5	20%	Linked to hepato-carcinoma
		N-sulfotransferase 4	100		1.2×10^4	15%	
10	+	Hsp73	73	70	3×10^7	23%	see 6
11	+	MGC:790	102	105	3.6×10^{10}	31%	Putative nuclease
		EBNA-2 co-activator	100		4.8×10^{10}	29%	Nuclear protein

Table 7: Proteins identified by MALDI-tof mass spectrometry. The corresponding number of the band on the gels, band intensity variation between G1 and S phases (\pm in S), name and theoretical molecular weight of the proteins (MW) are shown. The approximate MW of the cut band is also shown (MW cut). Highlighted in bold red are the cut sizes matching the theoretical sizes. The Mowse score is a scoring method used for peptide-mapping based on counting the number of measured peptide masses that correspond to calculated peptide masses present in the Mowse database allowing the identification of the protein (Pappin *et al* 1993). The percentage of coverage (Coverage) is also shown. n/a: not annotated.

1) Is septin a centrosomal protein?

Septins form a family of conserved proteins implicated in a variety of cellular functions. Septins are involved in cytokinesis (Kinoshita and Noda 2001). In *Drosophila melanogaster* they are transported to the cleavage furrow by pavarotti, a plus end motor protein (Adams *et al.* 1998). An immunoblot was performed to confirm the presence of septin in the centrosome preparations (Figure 15A). As shown on Figure 15A, the antibody recognised several septins, all of them enriched in the "S" fraction compared to "G1", confirming the presence of septins in the centrosome preparations. Since a protein present in these fractions could be a contaminant and, therefore not be associated with the centrosome, or associated with the centrosome in an unspecific manner, we decided to test the localisation of septin in Jurkat cells by immunofluorescence. In these cells, septin did not colocalise with the centrosomal marker γ -tubulin, suggesting that septin is not a centrosomal protein (Figure 15B). However, a centrosomal staining could be obscured by a strong cytoplasmic background staining, therefore we performed immunofluorescence experiments on isolated centrosomes to determine whether septin was located at the centrosome. "G1" and "S" centrosomes were co-stained with an anti-septin antibody and an anti α -tubulin antibody (Figure 15C). In both fractions, only a minority of centrosomes (~4%, n=205) were positive for septin staining. These results suggest that septin is not a *bona fide* protein, although present in the centrosome fractions.

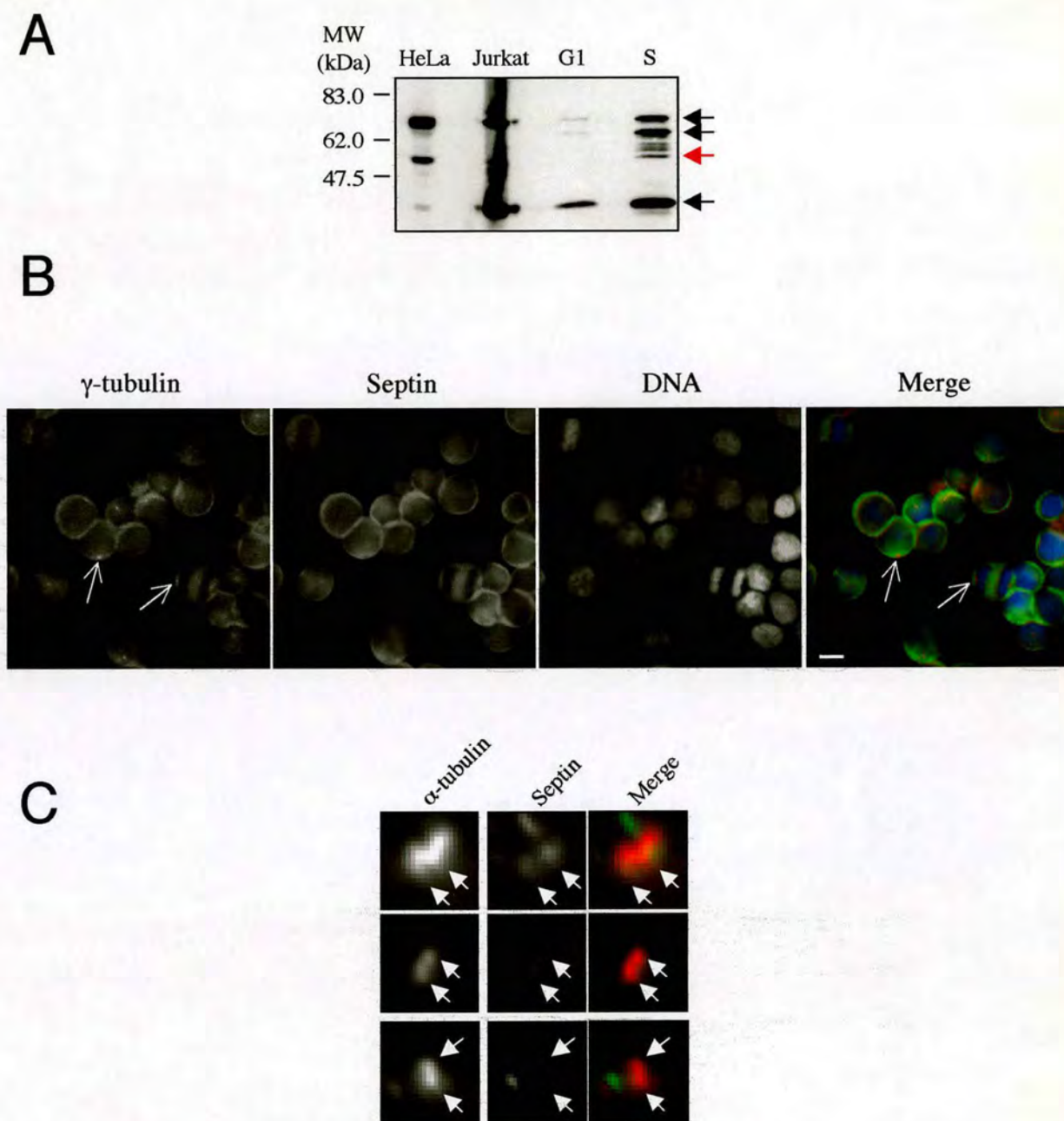


Figure 15: Septin, a centrosomal protein?

A, The pericentriolar material of centrosomes purified from unsynchronized cells (G1 lane) or cells arrested in S phase (S lane) were loaded together with protein extract from HeLa and Jurkat cells and probed with an anti-septin antibody. Immunological detection was performed using enhanced chemiluminescence. Black arrows indicate different isoforms detected by the antibody. Red arrow indicates a septin of the same molecular weight as the one identified by MALDI-tof. **B**, Methanol-fixed Jurkat cells immunostained with antibodies against γ -tubulin (red) and septin (green). Arrows point towards centrosomes. DNA was stained with DAPI (blue). Bar: 5 μ m. **C**, Immunofluorescence using anti- α -tubulin (red) and anti-septin (green) antibodies on isolated centrosomes. Arrows point to centrosomes.

No differences in septin signal intensity were observed between "G1" and "S" centrosomes, suggesting that the increase of septin signal intensity observed by immunoblotting did not result from a recruitment of septin at the centrosome at the onset of S phase. However, the fact that septin was detected in only 4% of the centrosomes, suggested that the protein could associate transiently with the centrosome. Since no differences were noted in septin signal between "G1" and "S" centrosomes, it is likely that this association occurs in G2 or M phases of the cell cycle.

2) Characterisation of the MCC1 protein

The *MCC1* (mutated in colorectal cancer 1) gene is located on chromosome 5q21 and was found mutated in several colorectal cancers (Kinzler *et al.* 1991). The MCC1 protein is 829 amino acids long and has an apparent molecular weight of ~100 kDa. The protein has a short region similar to the G protein-coupled m3 muscarinic acetylcholine receptor and contains several regions which have a high probability to form coiled-coil structures (Kinzler *et al.* 1991). MCC1 has been localised in the cytoplasm of NIH3T3 cells and has been proposed to play a role in the signaling pathway negatively regulating the G1/S transition, since overexpression of MCC blocks cell cycle progression from G1 to S phase (Matsumine *et al.* 1996).

We obtained the full-length human MCC1 cDNA from the IMAGE consortium (clone IMAGE n° 4111706) and expressed two portions of the protein in bacteria (MCC₉₈₇: amino acids 180 to 510, MCC₈₂₁: amino acids 523 to 797). The expressed polypeptides were subsequently used to immunise rabbits. Serum from one rabbit immunised with MCC₈₂₁ failed to detect any protein of the expected molecular weight

when used on immunoblot, and gave a staining reminiscent of intermediate filaments, suggesting a keratin contamination during the immunisation procedure (data not shown). A different antibody, raised against MCC₉₈₇, recognised a ~100 kDa protein likely to be MCC1 when used in immunoblotting experiments (Figure 16B). Immunofluorescence on HeLa cells using the same antibody stained the nucleus in interphase cells (Figure 16A). Speckles were also observed in the nuclei of stained cells. In metaphase cells, the protein was found associated with the chromatin. No centrosome signal was observed either in interphase or metaphase cells. To determine whether MCC1 was a centrosomal protein "G1" and "S" centrosomes were immunostained with an anti α -tubulin antibody and our MCC1 serum. We did not observe any MCC1 staining, suggesting that MCC1 does not localise to the centrosome (Figure 16C). Hence, the MCC1 protein does not seem to be a centrosomal protein, but rather a nuclear protein associated with the chromatin.

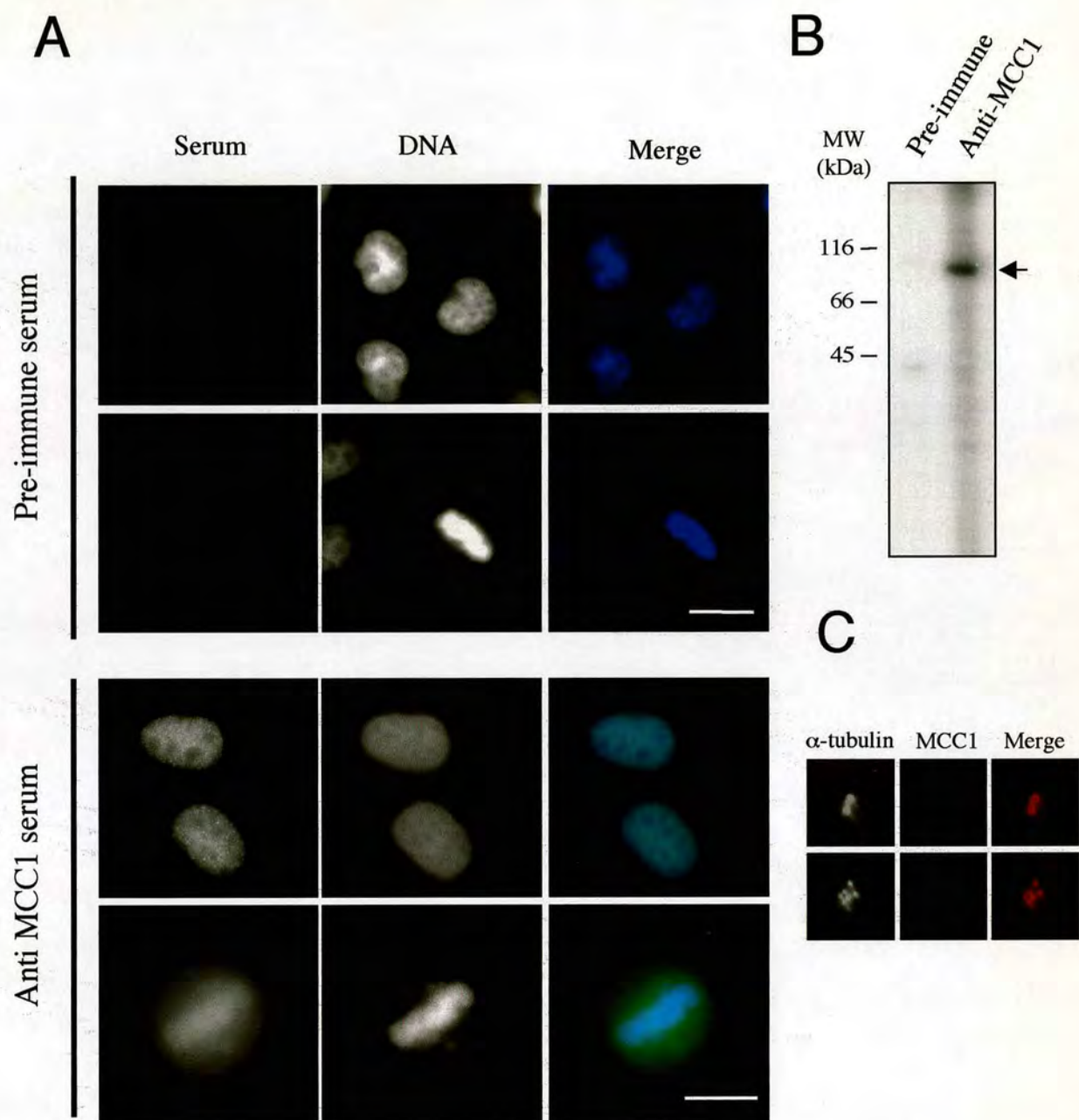


Figure 16: Characterisation of the MCC1 protein.

A, Methanol-fixed HeLa cells were stained either with pre-immune serum or with our anti-MCC1 serum (green). DNA was stained with DAPI (blue). Pictures were all taken at the same exposure. Bar 20 μ m. **B**, HeLa whole cell lysates were separated by SDS-PAGE, blotted and probed either with the pre-immune serum (pre-immune) or the anti-MCC1 serum (Anti-MCC1). Immunological detection was performed using enhanced chemiluminescence. **C**, Centrosomes were stained with anti- α -tubulin antibody (red) and anti-MCC1 serum (green).

3) Characterisation of the HCA66 protein

The *HCA66* gene is located on chromosome 17q11 and the HCA66 protein (accession number NP_060898.1) has recently been identified as an antigen in hepatocellular carcinomas, using human autoimmune sera (Jenne *et al.* 2001; Wang *et al.* 2002). The predicted protein sequence of HCA66 comprises 597 amino acids, with a theoretical molecular weight of 66 kDa. Database searches revealed highly homologous sequences from ESTs in mouse, *Drosophila*, *Anopheles*, and yeast (see Figure 17). Alignment of the HCA66 protein sequence from different species showed that the amino-terminal half of HCA66 (aa 1-202) is the most conserved region within the protein (61% homology between yeast and human), suggesting that this region plays a crucial role for the function of the protein (Figure 17). The HCA66 protein contains several TPR (tetra trico peptide repeat) motifs involved in protein-protein interactions. No other motifs were found using motif prediction software, and no functional data on HCA66 have been reported.

We have generated a cDNA clone encoding the full-length protein by reverse transcription of Jurkat cell RNA and PCR as described in Materials and Methods. We then expressed a recombinant portion of the protein (amino acids 86-365) in bacteria and raised an antibody in rabbits.

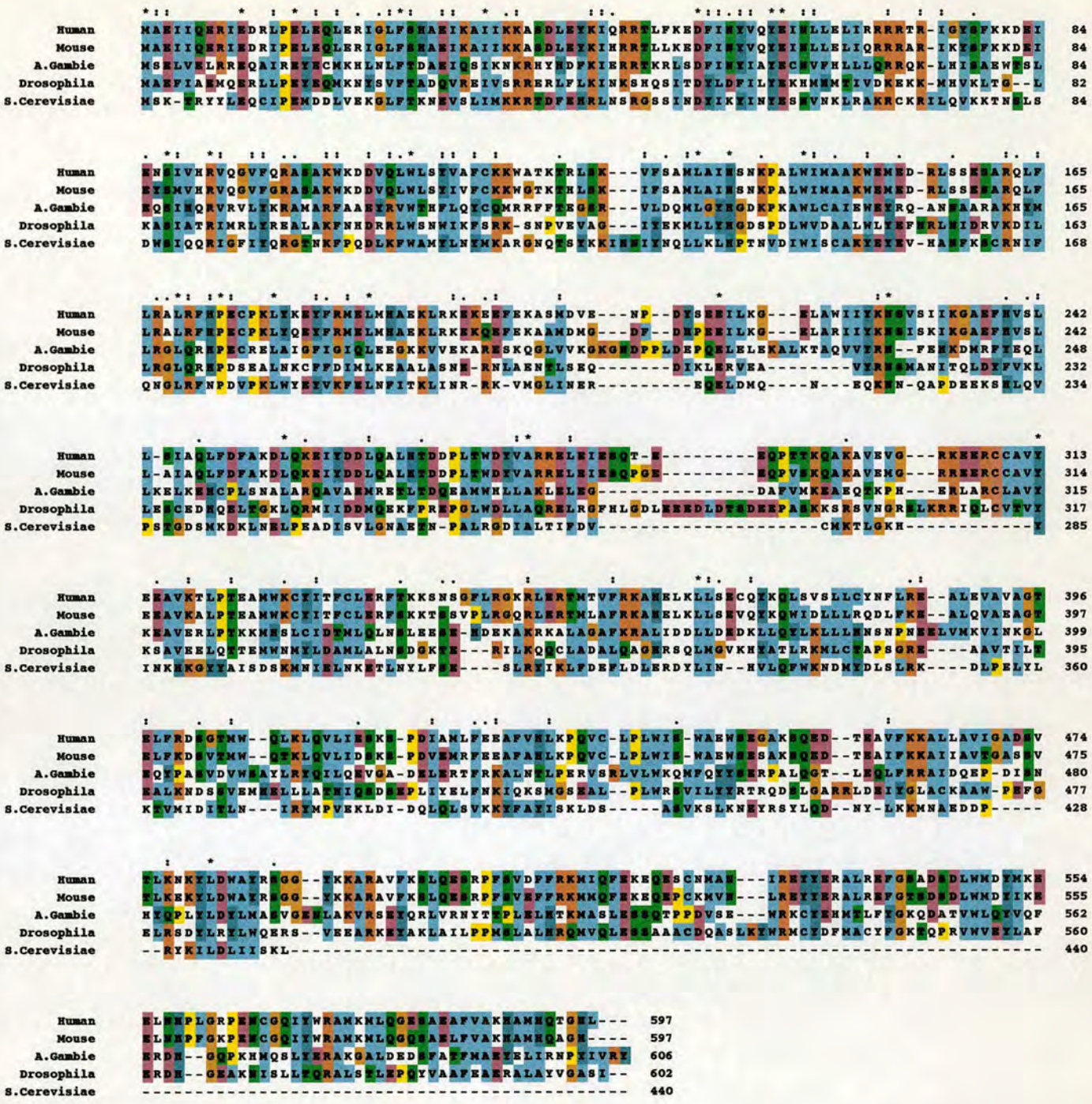


Figure 17: Sequence comparison between human, mouse, *Anopheles gambiae*, *Drosophila melanogaster*, and *Saccharomyces cerevisiae* HCA66 proteins realised with ClustalX.

Key to Clustal symbols, * - amino acid fully conserved, - : - strong group fully conserved, STA, NEQK, NHQK, QHRK, MILV, MIL, HY, FYW - weaker group fully conserved CSA, ATV, SAG, STNK, STAP, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY. Colouring of Clustal alignment, Blue: A F I L M V W, Purple: E D, Green: N Q S T, Yellow: P, Orange: G, red: K R, Turquoise: Y H, Salmon: C.

By immunoblot, the serum and the affinity purified antibody recognised mainly a ~60 kDa protein (Figure 18A). To confirm that the ~60 kDa protein was indeed HCA66, we cloned the HCA66 open reading frame into an eukaryotic expression vector containing a myc tag and transfected it into HeLa cells. As shown on Figure 18A, the tagged protein recognised by the anti-myc antibody had an apparent molecular weight of ~60 kDa migrating at the same position than the HCA66 signal. This suggests that our serum detects HCA66. Immunofluorescence microscopy on HeLa cells gave a staining pattern for HCA66 that was very peculiar. In interphase, the protein was mostly located in the nucleus. Speckles were also observed in the nuclei of interphase cells, suggesting the association of the protein with some structure such as Cajal bodies or nuclear bodies (Figure 18B and 18C). The nucleolar staining observed with the serum (Figure 18B) was seen only weakly with the affinity-purified antibody.

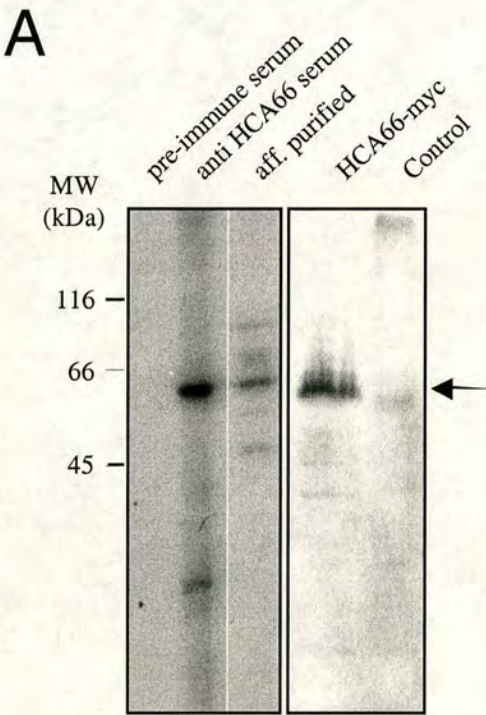
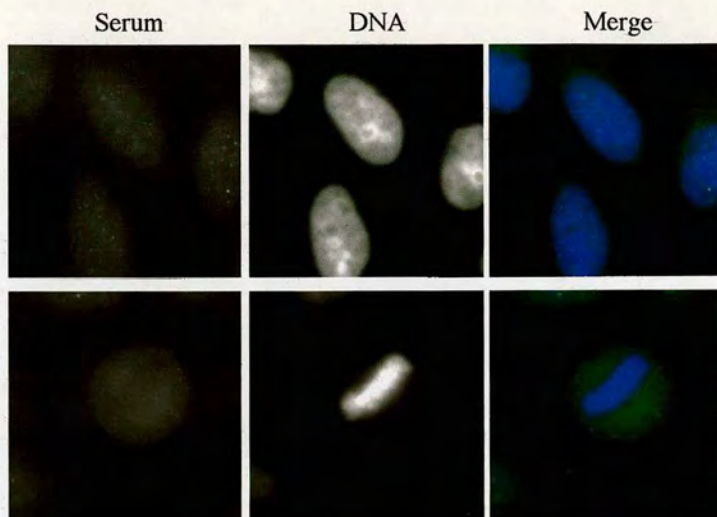


Figure 18: Characterisation of the anti HCA66 serum.

A, Immunoblotting on HeLa whole cell lysate using the pre-immune serum (pre-immune), the anti HCA66 serum and the anti HCA66 serum affinity purified over a HCA66 column (aff. purified) (Left panel). Whole cell lysate from HeLa cells transfected with HCA66-myc or control cells were separated by SDS-PAGE, transferred and probed with an anti-myc antibody (Right panel). Immunological detection was performed using enhanced chemiluminescence.

B

Pre-immune serum



anti HCA66 serum

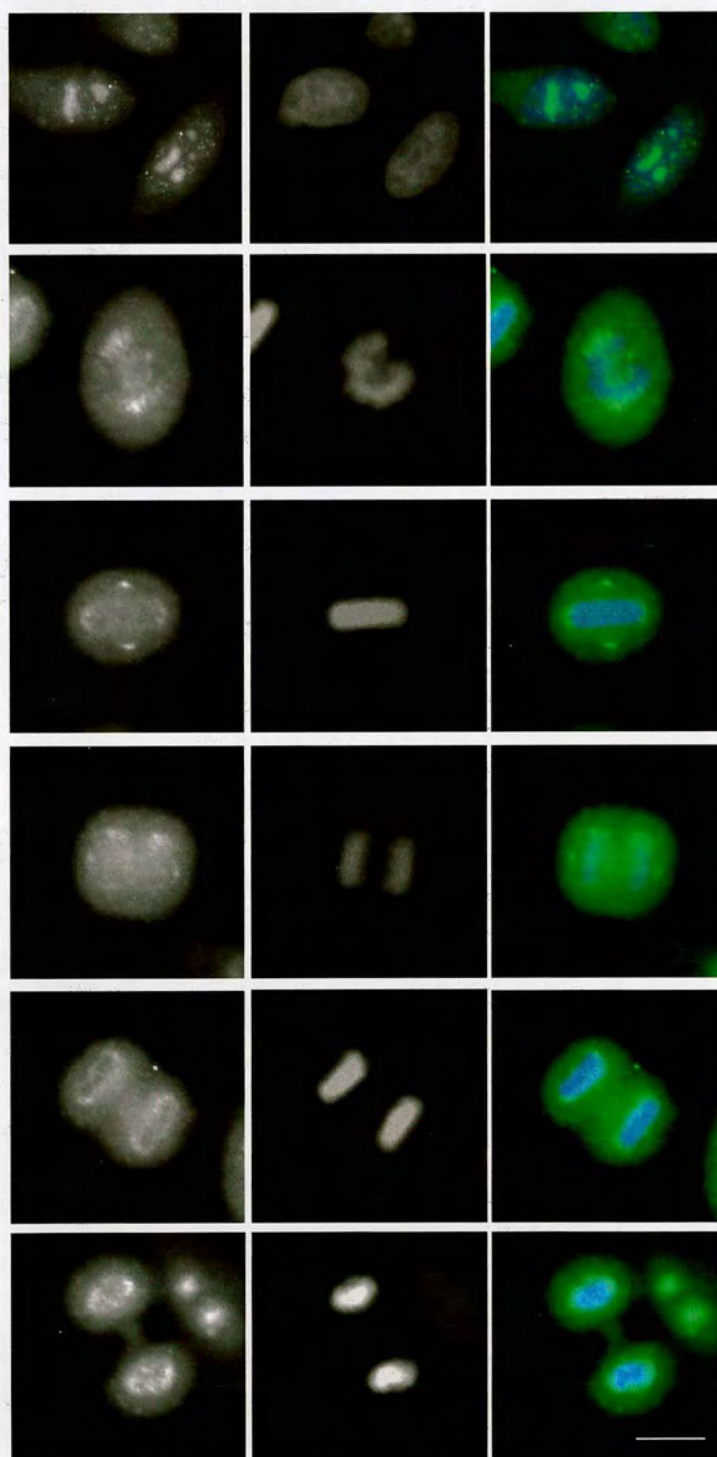


Figure 18 continued
B, Methanol-fixed HeLa cells were stained either with pre-immune serum or with anti HCA66 serum (green). DNA was stained with DAPI (blue). Pictures were all taken at the same exposure. Bar 20 μ m.

C

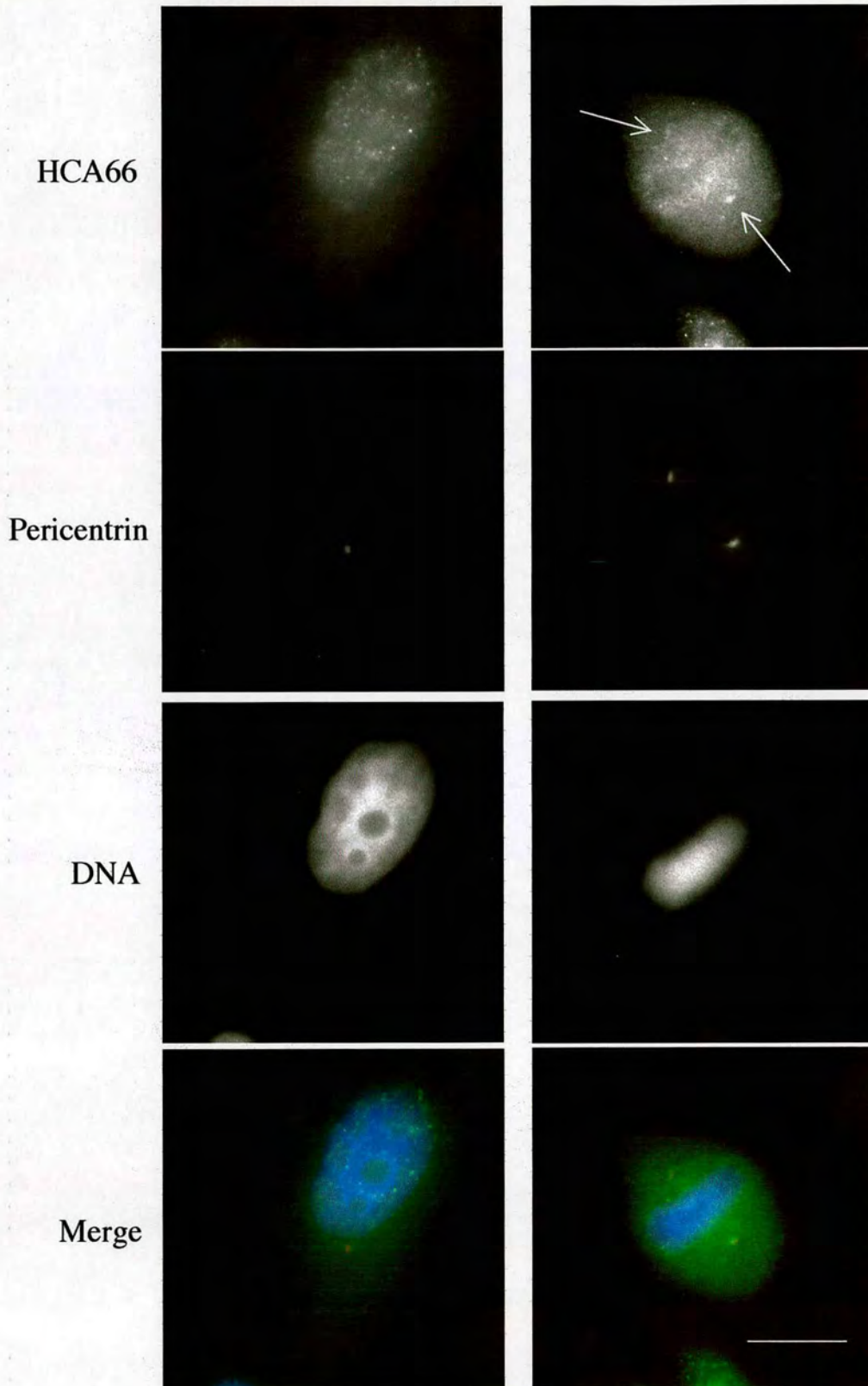


Figure 18 continued

C, Co-staining of pericentrin and HCA66 using the HCA66 affinity purified antibody. White arrows point to centrosomes. Bar 20 μm .

This was the only notable difference between the crude serum and the affinity-purified antibody. Upon entry into mitosis, HCA66 seemed to redistribute and associate with the centrosome / spindle pole as determined by co-staining with the centrosomal protein pericentrin (Figure 18C). Moreover, weak staining was still present at the chromatin periphery, suggesting that the protein is associated with the surface of the condensed chromosomes (Figure 18B and 18C). The centrosomal/spindle pole staining seemed most intense at metaphase (Figure 18B). Upon the onset of anaphase, the protein began to dissociate from the centrosome/spindle pole and was no longer associated with it in late anaphase/telophase (Figure 18B).

To determine whether HCA66 was associated with the centrosome, immunoblotting was performed on "G1" and "S" centrosomes. As shown in Figure 19A, a ~60 kDa band corresponding to HCA66 was detected by our antibody in G1 and S fractions. An ~80 kDa band was also detected in both fractions. To determine whether there was more HCA66 in "G1" than "S" centrosomes, the signal intensity was normalised to tubulin signal intensity (in S fraction, a doublet of α -tubulin was reproducibly obtained and the signal of both bands was added to determine the tubulin amount). Intensity of signal in "S" centrosomes was $\sim 1.75 \pm 0.22$ times higher compared to the HCA66 signal in the G1 fraction. To confirm this result and determine whether HCA66 was a centrosomal protein or was present in the fraction without being associated with the centrosome, we performed immunostaining experiments on isolated centrosomes (Figure 19B). As shown in Figure 19B, a signal was detected in both "G1" and "S" centrosomes. Moreover, quantification of the signals showed that staining in "G1" centrosomes (12.3 ± 4.3) was ~ 2.3 times weaker than staining in "S" centrosomes (27.33 ± 10.32) confirming results obtained by immunoblotting (Figure 19C).

These results strongly suggest that (1) HCA66 is indeed at the centrosome and (2) present in higher amounts in S centrosomes, suggesting a recruitment at the centrosome at the onset of S phase.

Interestingly, when used for immunoblotting experiments, our anti- α -tubulin antibody recognised two bands in "S" centrosomes (see Figure 17, see also Figure 10). The differences between the two bands might reflect differences in post-translational modifications of the tubulin (i.e. acetylation and/or glutamylation and/or phosphorylation) occurring at the G1/S transition. The ratio between the two bands was variable in different centrosome preparations (compare Figure 10 with Figure 17). In every case, the whole tubulin signal from both bands was taken into account when quantification of the signal was required.

E – Purification of centrosomes from cells in mitosis

We also wanted to compare the protein composition of "G1" centrosomes with the centrosomes of mitotic cells, therefore we attempted to isolate centrosomes from cells arrested in mitosis. We were not able to arrest Jurkat cells, HL60 or K562 cells (myeloid and erythro-leukemic cell lines respectively, chosen for the same reasons than Jurkat cells) in mitosis using microtubule depolymerising drugs such as nocodazole or colcemid, suggesting that these cells lack the spindle check-point. To bypass this problem, we tried to reconstitute a functional centrosome from DT40 (a chicken B lymphoma cell line) salt-stripped centrosomes and concentrated extract from Hela S3 cells in interphase or arrested in prometaphase (>75% mitotic cells; H1 kinase activity of the extract was tested, data not shown). This would have the advantage to discriminate between the

protein recruited to the reconstituted centrosome (of human origin) from the one present in the insoluble fraction (of chicken origin). Although, we were able to reconstituted a functional centrosome on coverslips (data not shown), we were unable to resolublise the KI-insoluble fraction in the extract and could not compare the composition of centrosomes from cells in mitosis with centrosomes purified from cells in other cell cycle stages.

In the past, various centrosomal proteins were described to localise in a broad, crescent-shaped area of the spindle pole when cells entered mitosis or meiosis. In particular in acentriolar spindles of mouse oocytes, centrosomal proteins such as pericentrin were found in a large "cap-like" region associated with minus ends of spindle microtubules (Doxsey *et al.* 1994). This staining was reminiscent of the distribution of a microtubule binding protein that stabilises the spindle pole, NuMA (Merdes *et al.* 1996). We were interested to determine, whether direct binding between centrosomal proteins at the microtubule ends existed in the mitotic spindle. We therefore investigated 4.1R, a protein that had previously been shown to localise to the centrosome and the spindle pole, and that was found to interact with NuMA (Krauss *et al.* 1997b; Mattagajasingh *et al.* 1999).

II 4.1R a multi-faceted protein

The centrosomal localisation of 4.1R protein was first determined by electron microscopy and immunolocalisation (Krauss *et al.* 1997b). However, this study did not determine which 4.1R isoforms were located at the centrosome. So far, only the 4.1R¹³⁵ isoform has been located to the centrosome using immunofluorescence (Hung *et al.* 2000).

A - Is 4.1R a centrosomal protein?

We decided to investigate which of the 4.1R isoforms was present at the centrosome. We performed immunoblotting on purified centrosomes from Jurkat cells using an anti 4.1R antibody directed against the carboxy-terminal domain of the protein and recognising 4.1R¹³⁵ and 4.1R⁸⁰ (generous gift of Dr. A. Baines (Scott *et al.* 2001)). As seen on Figure 20, Jurkat cells expresses the 80 and 135 kDa isoforms of 4.1R. To our surprise, the antibody did not detect any band in the centrosome fraction suggesting that the 4.1R isoforms recognised by this antibody were not *bona fide* centrosomal proteins.

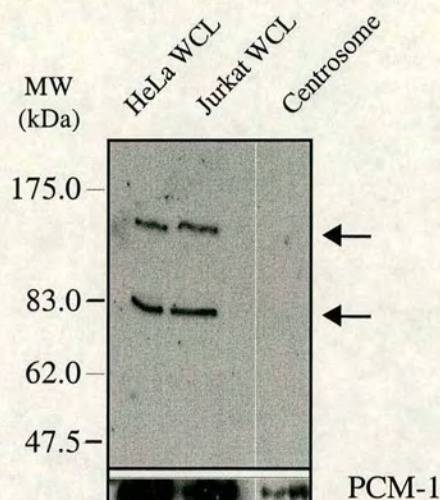


Figure 20: 4.1R is not detected on the centrosome.

Whole cell lysates from HeLa (HeLa WCL) and Jurkat (Jurkat WCL) cells and purified centrosomes from Jurkat cells were immunoblotted with an anti-4.1R antibody (generous gift of A. Baines) and an anti-PCM-1 antibody. Immunological detection was performed using enhanced chemiluminescence.

B – Characterisation of polyclonal antibodies raised against 4.1R

We decided to raise antibodies against the *Xenopus laevis* 4.1R¹³⁵ isoform (x4.1R¹³⁵). cDNA encoding amino acids 222 to 531 (4.1M) and 530 to 801 (4.1C) of x4.1R¹³⁵ (Figure 21) were expressed in bacteria, purified and used to immunise rabbits (4.1M: rabbits 1044 and 1045; 4.1C: rabbits 1046 and 1047).

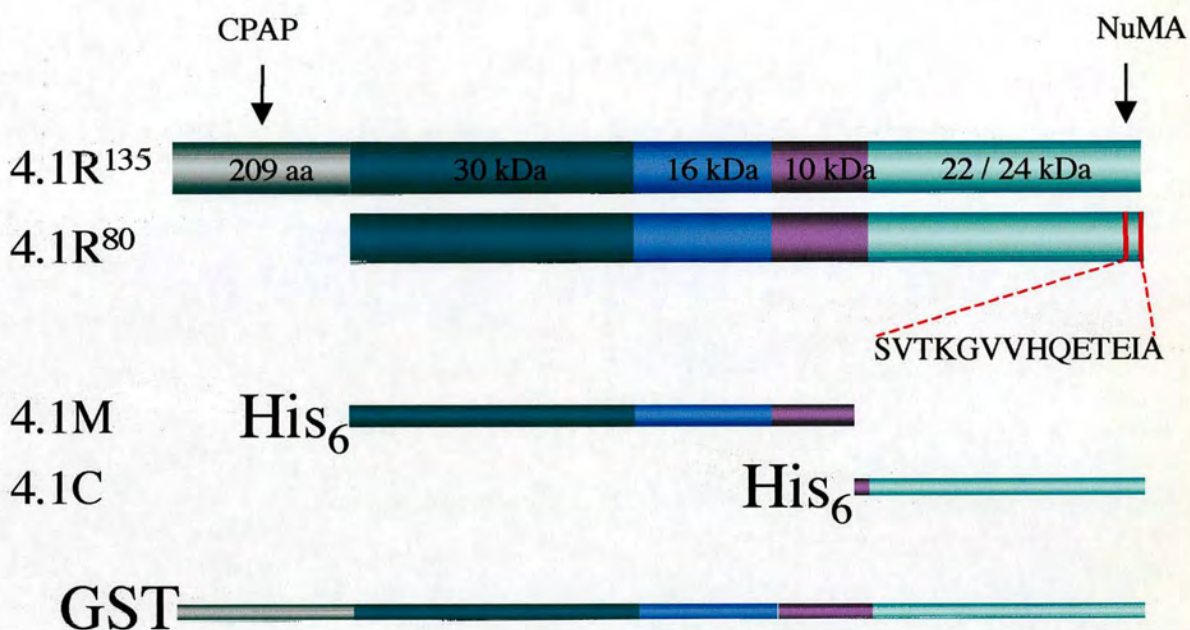


Figure 21: The different polypeptides used to raise antibodies against 4.1R.

Schematic representation of the two main isoforms of the 4.1R protein, 4.1R¹³⁵ and 4.1R⁸⁰, and the different polypeptides (fusion proteins 4.1M, 4.1C, GST-full-length or synthetic peptide) used to raise antibodies against the *Xenopus laevis* isoforms of the protein. CPAP and NuMA interaction domains are shown.

Sera from rabbits immunised with the same antigen gave the same results, therefore only sera 1044 and 1047 are described thereafter. As shown in Figure 22A, both sera reacted against the recombinant full-length protein by immunoblot. However, in whole cell lysate from HeLa cells none of the sera recognised a protein of the expected molecular weight (i.e. 80 kDa or 135 kDa). Instead, serum 1044 detected a ~37 kDa protein, whereas serum 1047 detected a ~50 kDa protein (Figure 22A). These antibodies were used by immunofluorescence on HeLa cells. As shown on Figure 22B, serum 1044 gave a diffuse cytoplasmic staining in interphase and mitotic cells, although some staining of the spindle could also be seen. Serum 1047 gave a diffuse cytoplasmic staining both in interphase and mitotic cells (Figure 22B).

Neither of these two antibodies detected a centrosomal isoform of 4.1R and therefore they could not be used for our experiments. Another antigen was chosen to raise antibodies against a centrosomal isoform of 4.1R.

A GST fusion of x4.1R¹³⁵ was expressed, purified and used to immunise two rabbits (1631 and 1632). Only serum 1632 is described below since both sera gave the same results.

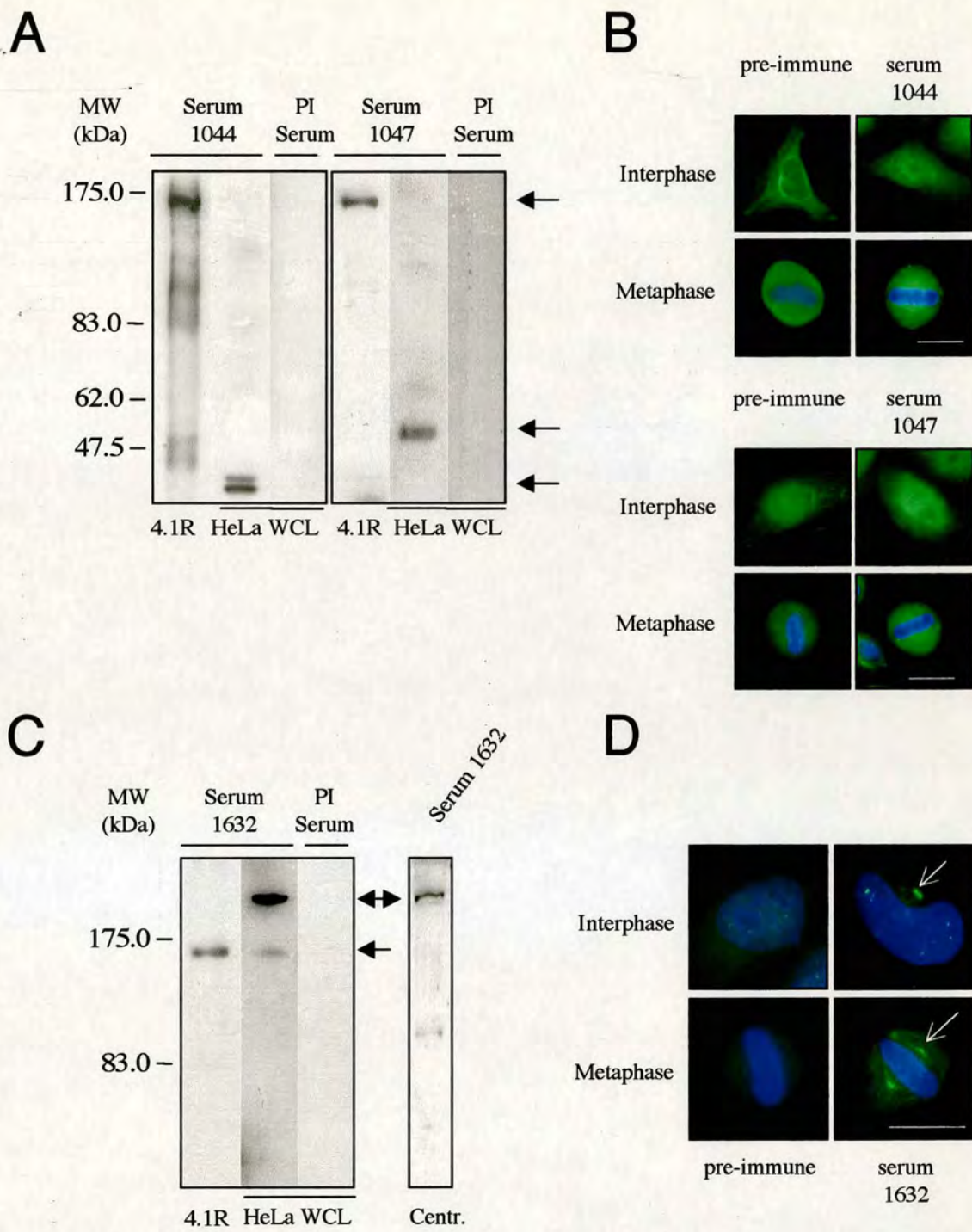


Figure 22: Characterisation of the different anti-4.1 polyclonal antibodies.

A, 4.1R¹³⁵His-tagged fusion protein and HeLa whole cell lysates (HeLa WCL) were run on a 7.5% polyacrylamide gel, blotted and incubated with sera 1044, 1047 and the respective pre-immune sera (PI serum). **B**, Methanol-fixed HeLa cells stained with sera 1044, 1047 and the respective pre-immune sera. An interphase and a metaphase cell are shown. **C**, 4.1R¹³⁵His-tagged fusion protein and HeLa whole cell lysates (HeLa WCL) separated by SDS-PAGE, blotted and incubated with serum 1632 and the respective pre-immune serum (PI serum). Serum 1632 was also used by immunoblotting on isolated centrosomes (centr.). **D**, Methanol-fixed HeLa cells stained with serum 1632 and the respective pre-immune serum. An interphase and a metaphase cell are shown. Arrows point to centrosomes. Immunological detection in A and C was performed using enhanced chemiluminescence. Bars in B and D, 20 μ m.

Our serum detected the full-length recombinant protein (His₆-tagged) when used on immunoblot (Figure 22C). In HeLa whole cell lysates, serum 1632 reacted with a major band corresponding to a protein of apparent molecular weight of ~210 kDa. The sera also detected another protein of ~140 kDa although with a weaker reactivity (Figure 22C). These two proteins might be different isoforms of the 4.1R protein. As expected, when used by immunostaining the serum gave a clear centrosomal staining both in interphase and mitotic HeLa cells (Figure 22D). To determine which of the two proteins was localised at the centrosome, we performed an immunoblotting experiment on isolated centrosomes. As shown in Figure 22C, the 1632 antibody only detected the 210 kDa band. Taken together these results suggest that a 210 kDa isoform of the 4.1R protein is located at the centrosome.

C - A mouse polyclonal antibody recognising 4.1R

We also tried to isolate a hybridoma clone producing a monoclonal antibody against the centrosomal/spindle pole isoform(s) of 4.1R.

The 4.1C construct was used to immunise a BALB/c mouse. The mouse bleed showed a typical 4.1R staining in chicken DU249 cells along with a centrosomal staining in interphase and mitotic cells (Figure 23A).

When used in immunoblotting experiments the bleed recognised two proteins of 135 and 80 kDa in HeLa whole cell lysates and of 145 and 135 kDa in chicken DT40 whole cell lysates (Figure 23B). In order to isolate a monoclonal antibody, a hybridoma of B cells from the immunised mouse and myeloma cells was made by Dr A. Merdes.

However, we were not able to isolate a stable clone expressing an antibody with the same immunofluorescence and immunoblotting patterns as the mouse bleed.

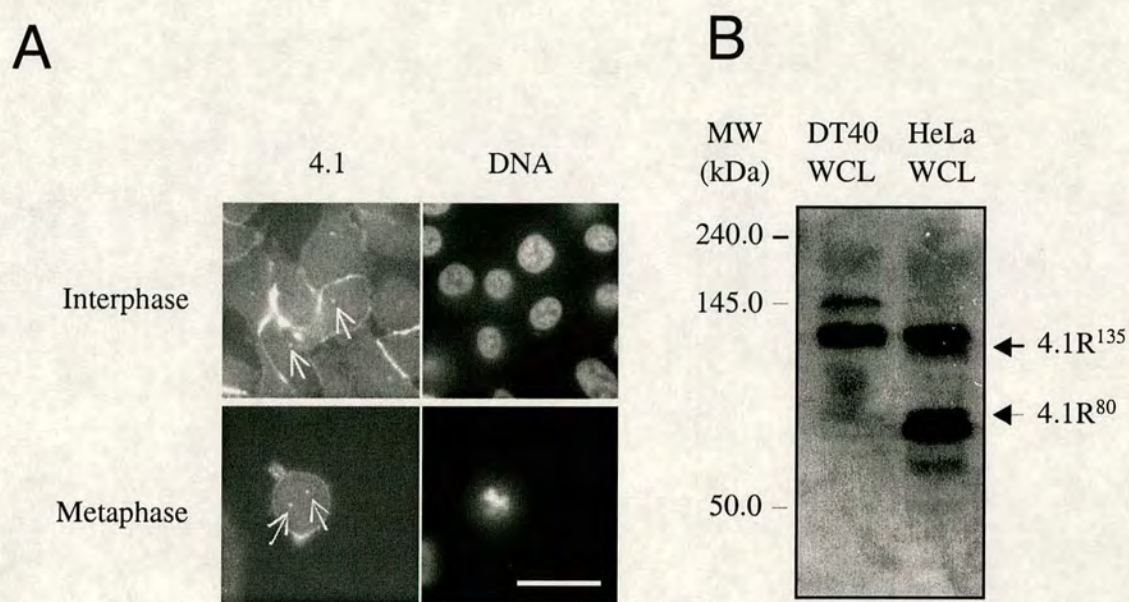


Figure 23: Characterisation of an anti-4.1R mouse polyclonal antibody.

A, Chicken DU249 cells were stained with mouse anti-4.1R serum. Cells in interphase and in metaphase are shown. The corresponding DNA staining with DAPI is shown. Note the plasma membrane staining at the sites of cell-cell contact, and the centrosome staining (arrows). Bar 20 μ m. **B**, DT40 and HeLa whole cell lysates (WCL) blotted and probed with the mouse anti-4.1R serum. Immunological detection was performed using enhanced chemiluminescence.

D - β -tubulin and 4.1 share a structural homology

Since the results we obtained with our antibodies were unsatisfactory, we raised an antibody against a synthetic peptide of the last 14 amino acids of x4.1R¹³⁵ (amino acids 788 to 801; see Materials and Methods). Two rabbits (1781 and 1782) were immunised and both gave a positive immune response to the peptide, as seen on dot blots

(Figure 24A), whereas the reactivity against a control peptide from *Xenopus* NuMA was very low (Figure 24A). The two sera were further characterised by immunoblotting and immunostaining on tissue culture cells, and both gave identical results. For this reason, only serum from rabbit 1782 is described hereafter. When used on HeLa cells by immunofluorescence, the antibody stained a structure reminiscent of microtubules in interphase and the mitotic spindle in mitosis (Figure 24B). Moreover, when cells were treated with nocodazole, a microtubule depolymerising drug, staining with the anti-4.1R serum was restricted to the centrosomal area (Figure 24C), whereas the pre-immune serum did not detect any centrosomal signal. Double immunofluorescence with our serum and an anti α -tubulin antibody confirmed that the peptide antibody against 4.1R stained indeed the microtubule network (Figure 24D). These results suggested that 4.1R could be associated with microtubules, which would be consistent with previous observations (Correas and Avila 1988). Alternatively, the observed pattern may be due to a cross-reactivity of the antibody with tubulin or a microtubule associated protein. To discriminate between these two possibilities, we performed additional experiments.

When immunoblots were performed, the serum showed a strong response against a recombinant full-length x4.1R¹³⁵, whereas the pre-immune serum did not. Moreover, the serum predominantly reacted against a 55 kDa band in whole cell lysates from *Xenopus laevis* A6 cells and recognised pure brain tubulin, suggesting a cross-reactivity of the antibody with tubulin (Figure 25).

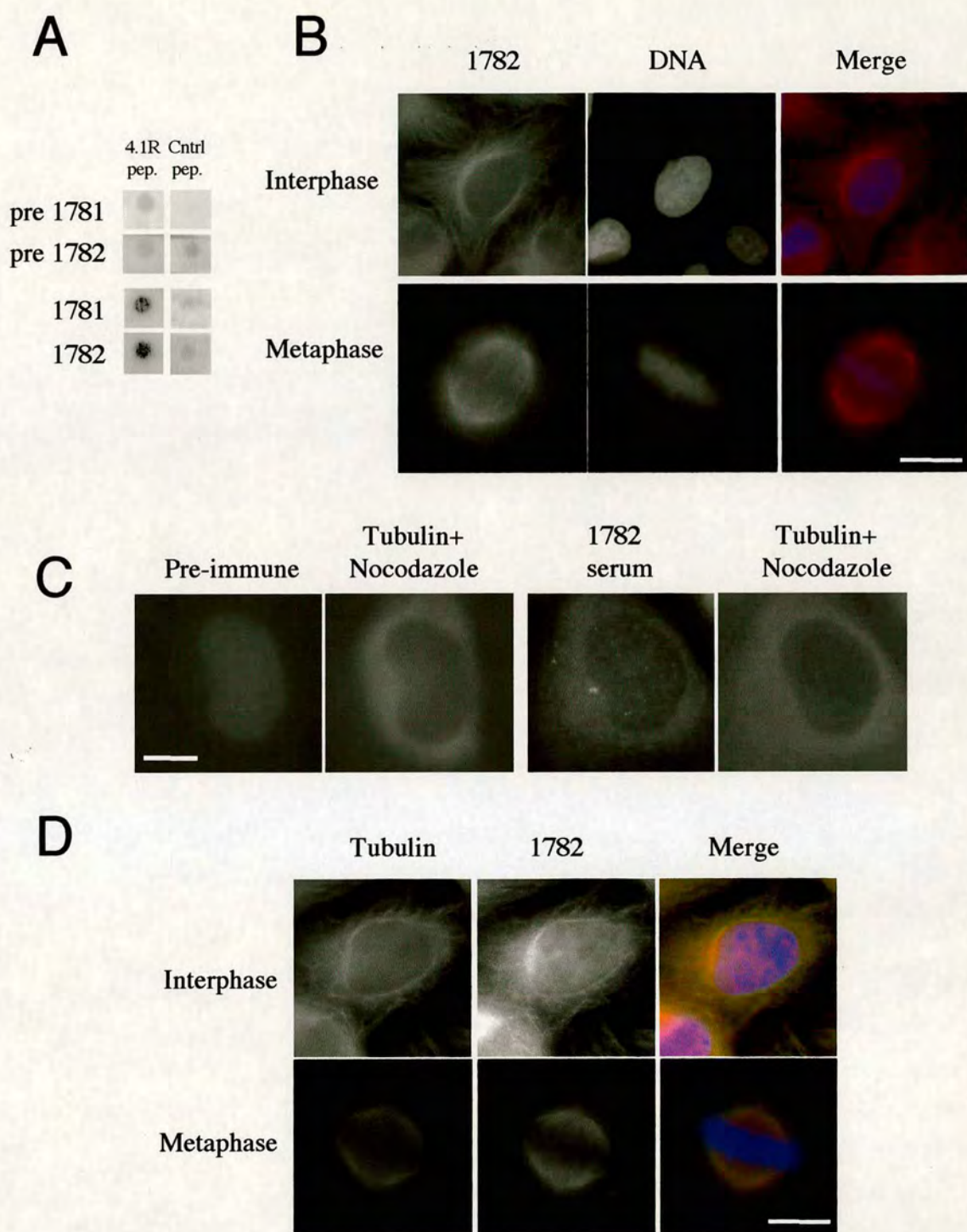


Figure 24: A serum raised against a 4.1R C-terminal peptide stains the microtubule network.

A, A 4.1R peptide (4.1R pep.) and a control peptide (cntrl pep.) of the tail domain of *Xenopus* NuMA were transferred onto nitrocellulose membranes using a dot-blotter and the membranes were probed either with pre-immune sera or immune sera from rabbits 1781 and 1782. **B**, Methanol-fixed HeLa cells were incubated with the 1782 serum (red) and DAPI to stain DNA (blue). Examples of a cell in interphase and a cell in metaphase are shown. **C**, HeLa cells treated with nocodazole and stained with an anti-tubulin antibody and either pre-immune serum or 1782 serum. **D**, Double immunostaining of HeLa cells with a monoclonal anti-tubulin antibody (green) and 1782 serum (red). DNA is stained with DAPI (blue). An interphase and a mitotic cell are shown. Bars in B, C and D, 20 μ m.

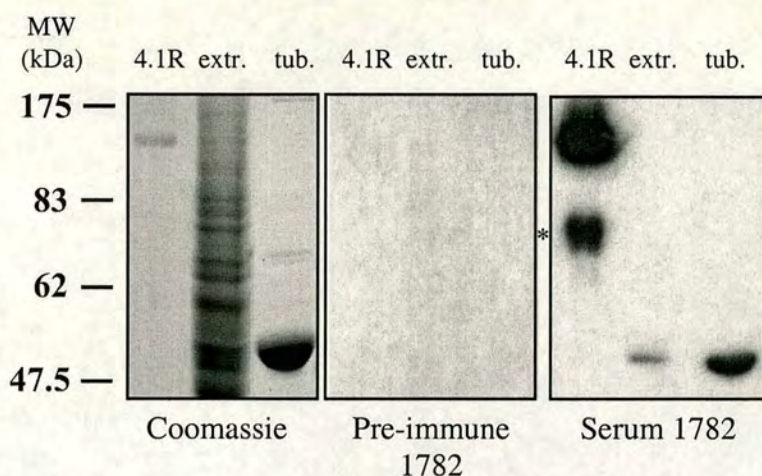


Figure 25: 1782 serum raised against a 4.1R C-terminal peptide recognises tubulin.

Polyacrylamide gels containing recombinant full-length 4.1R protein (lane 4.1R), protein extracts from *Xenopus* A6 cells (lane extr.), and purified bovine brain tubulin (lane tub.) were stained with a coomassie blue solution (Coomassie), or blotted onto nitrocellulose and probed with rabbit pre-immune serum 1782, or serum 1782. * indicates a degradation product of the recombinant 4.1R protein reacting with our serum. Immunological detection was performed using enhanced chemiluminescence.

Identical properties were observed in the sera from both rabbits, suggesting that the detection of tubulin was due to a cross-reactivity with the 4.1R epitope, rather than due to independent antibody titres against two proteins, as occasionally occurring in auto-immune sera. However, in order to distinguish between these two possibilities, we affinity purified the serum on recombinant x4.1R¹³⁵ protein. Immunofluorescence and immunoblotting experiments showed that the reactivity against tubulin was preserved in the anti-4.1R antibody (Figure 26A, B), indicating that the same antibody cross-reacted both with 4.1R and tubulin. Consistently, serum that was affinity purified on pure brain tubulin also recognised the recombinant x4.1R¹³⁵ protein by immunoblotting (Figure 26A). To our surprise, these affinity-purified antibodies were both specific for the β -tubulin isoform, since they did not recognise recombinant α -tubulin expressed in bacteria (Figure 26A).

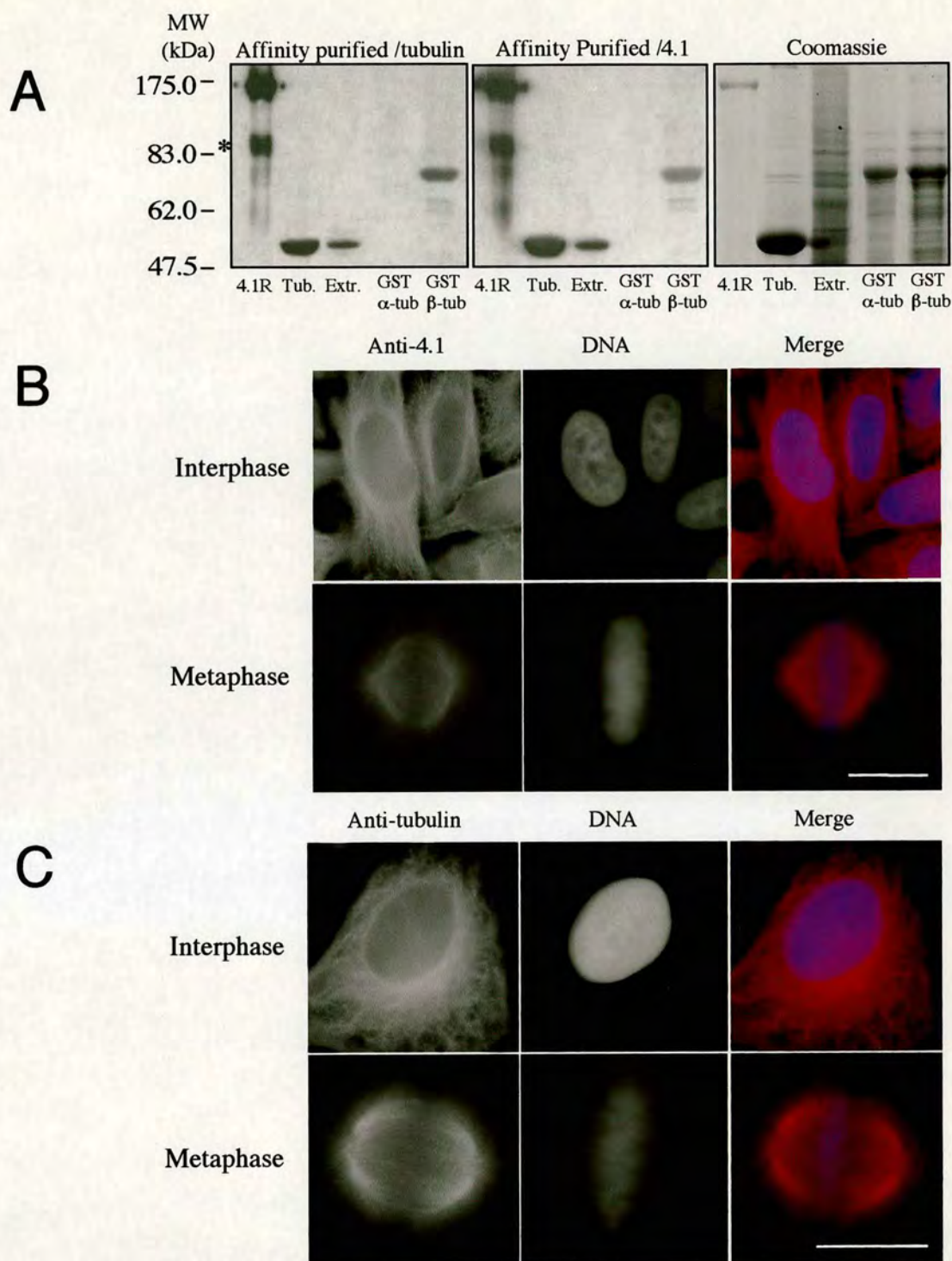


Figure 26: Anti-4.1R antibodies cross-react with β -tubulin.

A, Coomassie staining of a protein gel loaded with full-length 4.1R protein (4.1R), brain tubulin (Tub.), *Xenopus* egg extracts (Extr.), and GST-tagged recombinant α -tubulin (GST α -Tub) or β -tubulin (GST β -Tub), respectively. Note the increased molecular weight of the recombinant forms of tubulin after addition of the GST-tag. Identical gels were blotted and probed with 4.1R peptide antibodies, previously affinity purified from brain tubulin (left) or recombinant 4.1R (middle). * denotes the position of a degradation product of 4.1R. Immunological detection was performed using enhanced chemiluminescence. **B** and **C**, The same affinity purified antibodies were used for immunofluorescence staining of HeLa cells. Interphase and metaphase cells are shown. Affinity purified antibody over 4.1R is shown in **B**, and affinity purified antibody over tubulin is shown in **C**. DNA was stained using DAPI (blue). Bars in **B** and **C**, 20 μ m.

An alignment of the 4.1R peptide sequence with α - and β -tubulin sequences revealed 29% identity within amino acids 183 to 196 of β -tubulin, but no significant similarity with α -tubulin (Figure 27). The specified region is located at the end of helix 5 in β -tubulin, and is exposed to the outer surface of the microtubule polymer (Nogales *et al.* 1999).

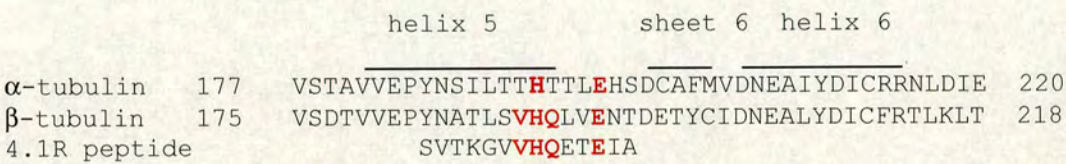


Figure 27: 4.1R peptide and β -tubulin share some homology.

Alignment of the C-terminal 4.1R peptide with amino acids 177 to 236 of α -tubulin and the corresponding region in β -tubulin (amino acids 175 to 234). The positions of α -helices and β -sheets in tubulin are indicated. Identical amino acids are highlighted in red.

Although the degree of similarity between the 4.1R peptide and β -tubulin is weak, our results open the possibility that the antibody cross-reactivity reflects a structural homology between the helix 5 of the β -tubulin molecule and amino acids 788 to 801 of x4.1R¹³⁵.

Although these antibodies reacted against an isoform of 4.1R located at the centrosome, we could not use them as a tool to study the biological function of this protein, because the tubulin cross-reactivity largely obscured the centrosome specific 4.1R staining.

Table 8 summarises the different attempts and strategies we used to raise an antibody against 4.1R¹³⁵.

Name	Antigen	Type	Immunoblot	Immunofluorescence
1044	Amino acids 222 to 531 of x4.1R ¹³⁵ (4.1M)	Rabbit Polyclonal	~37 kDa protein	Diffuse cytoplasmic.
1045				
1046	Amino acids 530 to 801 of x4.1R ¹³⁵ (4.1C)	Rabbit Polyclonal	~50 kDa protein	Diffuse cytoplasmic.
1047				
1631	GST x4.1R ¹³⁵	Rabbit Polyclonal	~210 kDa and ~140 kDa proteins	Centrosomes.
1632				
Mouse Serum	Amino acids 530 to 801 of x4.1R ¹³⁵ (4.1C)	Mouse Polyclonal	~135 kDa and 80kDa proteins	Centrosomes and plasma membrane.
1F11		Mouse Monoclonal	~ 200 kDa protein	Centrosomes (metaphase- telophase).
1781	Amino acids 788 to 801 of x4.1R ¹³⁵	Rabbit Polyclonal	β -Tubulin	Microtubules, and centrosomes.
1782				

Table 8: Antibodies raised using different 4.1R antigens. The molecular weight (or the name of the protein when identified) recognised by immunoblot are shown. The pattern observed by immunofluorescence is described.

DISCUSSION

The goal of our study was to identify centrosomal proteins potentially involved in the initiation of centrosome duplication or the control of the G1/S transition by the centrosome. Proteins from the pericentriolar material of "G1" and "S" centrosomes were separated using one-dimensional gels (1D-gels) and analysed by MALDI-tof mass spectrometry. Eight proteins were found differentially localised between "G1" and "S" centrosomes.

Changes occurring at the G1/S transition can be both qualitative and quantitative. Using a 1D-gel protein separation technique did not allow us to distinguish qualitative changes occurring in the PCM (for instance, phosphorylation events mediated by cdk2-cyclin A or E), limiting us to quantitative modifications. However, by using this technique, we were able to identify several new proteins, which might have a role in centrosome function or centrosome duplication, and decided to concentrate our efforts in investigating these proteins. A way to see qualitative changes such as phosphorylation of specific proteins of the PCM would have been to perform two-dimensional electrophoresis (2D-gel) analysis. Although we attempted to use this technique, we soon realised that significantly more cell material would have to be processed to allow detection of protein spots in two dimensions, exceeding the capacity of our facility.

We chose to compare an unsynchronised population of cells with a population arrested in early S phase. The unsynchronised population, although mainly in G1,

contained 25% of cells in S phase (Figure 9). This high proportion of cells in S phase made the detection of small quantitative changes harder. It might also explain why we found only 1.75 times more HCA66 at the PCM of "S" centrosomes by immunoblotting and an average of 2.3 times more by immunofluorescence, since a quarter of the "G1" centrosomes were actually "S" centrosomes. Therefore, the unsynchronised population of cells should possess more HCA66 than a 100% G1 population. Thus, it is likely that the S/G1 ratio of HCA66 was underestimated and that there is more HCA66 at the "S" centrosome than observed.

In our screen, we did not find NPM/B23 which is described to leave the centrosome at the beginning of S phase upon phosphorylation by cdk2 (Okuda *et al.* 2000, see Introduction). At least two possibilities can explain this. Firstly, differences in expression of the protein might have been too weak to be detected, or were masked by the presence of other proteins of a similar molecular weight. Secondly, it is possible that cells were blocked at a stage before NPM/B23 left the centrosome. Indeed, aphidicolin is an inhibitor of DNA polymerase δ , an enzyme involved in the initiation step of DNA replication. Therefore, cells were blocked at the very beginning of S phase, possibly prior to the separation of NPM/B23 from the centrosome. Immunoblotting experiments using an anti-NPM/B23 antibody would allow us to determine whether the protein was still located at the "S" centrosome.

Similarly, other changes in the protein composition of the PCM from "S" centrosomes might not have been detected due to the very early stage of S phase the cells were blocked in, which may explain the relatively small number of differences detected. As mentioned above, we could not detect qualitative changes, which also accounts for

this small number of differences. It is possible that the use of another technique to arrest the cells later in S phase would have given different results, and perhaps more candidates.

The comparison of the PCM composition in the other cell cycle phases (i.e. G2 and mitosis) would also have been of great interest. The attempt we made to compare the composition of the PCM between interphase and mitotic cells have so far been unsuccessful because the cell system used here, Jurkat cells, could not be arrested in mitosis using standard procedures of microtubule depolymerisation. Attempts to bypass this problem, by reconstituting a functional centrosome from potassium iodide-stripped DT40 centrosomes and concentrated extract from interphase or mitotic HeLa S3 cells, were unsuccessful due to the insolubility of salt-stripped centrosome in the extract.

The eight proteins we found when comparing the PCM composition of "G1" and "S" centrosomes all belong to different classes of protein. The nuclear proteins (MCC1, EBNA-2 and may be hnRNP L) and the one associated with the translational machinery (Eukaryotic translation initiation factor 4 α) were presumably contaminants. Among the proteins identified, only one, hsp73, was previously described as a centrosomal protein (Brown *et al.* 1996a). Pyruvate kinase is an enzyme of glycolysis, septin has been implicated in cytokinesis (see Kinoshita and Noda 2001 for review) and HCA66 is an uncharacterised protein linked to an hepatocarcinoma (Wang *et al.* 2002).

Hsp73 is recruited at the centrosome at the beginning of S phase

Hsp73 is a molecular chaperone produced in increasing amounts after a thermal stress to repair or replace proteinaceous components. It has been shown that hsp73 facilitates the recovery of the centrosomal structure after heat-shock. Furthermore, hsp73 is present at the centrosome throughout the cell cycle in unstressed cells (Brown *et al.* 1996a). Other chaperones such as tailless complex polypeptide-1 (TCP-1) or hsp90 were also described at the centrosome (Brown *et al.* 1996b; Lange *et al.* 2000; de Carcer *et al.* 2001). Molecular chaperones have been proposed to facilitate the movement of proteins from and to the centrosome as well as to promote spatial changes in the organisation of the PCM which occur during centrosome duplication (Andersen 1999). This would be consistent with the recruitment of hsp73 at the beginning of S phase, which would be required to modulate centrosome assembly. Alternatively, hsp73 could be required to stabilise centrosomal proteins throughout the cell cycle and at the time of centrosome duplication. This stabilisation effect was previously described in *Drosophila*, for hsp90, where it is required for polo kinase activity (de Carcer *et al.* 2001).

A centrosomal role for pyruvate kinase?

We identified pyruvate kinase (bands n°2 and n°5) as a potential centrosomal protein. Quantitatively, there is less pyruvate kinase in the "S" fraction than in the "G1" one. Being

centrally located at the minus-ends of the microtubules the centrosome could also serve as a "meeting place", bringing regulatory molecules close to their substrate to control the specificity of their functions. Thus, pyruvate kinase could be at the centrosome only to phosphorylate its substrates and may not play a role in centrosome duplication or cell cycle progression *per se*. Moreover, one can not exclude that this protein was merely a contaminant of the centrosome preparation. However, enolase, another enzyme of the glycolytic pathway, was also described at the centrosome and more recently a phosphorylated form of glycogen synthase kinase-3 was located at the centrosome, and the spindle pole (Johnstone *et al.* 1992; Wakefield *et al.* 2003). Taken together, these data point towards a role at the centrosome for several enzymes involved in the glucose metabolism.

The particular case of septin

Septin was identified in band n° 1 along with glutamate dehydrogenase (Table 6 and Figure 14). As mentioned in the introduction, centrosomes are required for the completion of cytokinesis through a yet undetermined mechanism. Septin is also involved in cytokinesis, therefore presence of septin at the centrosome could provide an explanation for the requirement of the centrosome for cytokinesis. Although present in the centrosome-enriched fraction from G1 and S cells, we detected a centrosomal septin signal by immunofluorescence on purified centrosomes only in very few cases (4%). The same results and signal intensity (data not shown) were obtained for "G1" and "S" centrosomes by immunofluorescence. Therefore, the differences observed in the silver stained gel and by immunoblotting may be due to soluble septin contaminating the centrosome fractions. It is also possible that the 4% of centrosomes with associated septin do not come from G1 or S

phase cells, but instead from G2 or mitotic cells which are present at this low percentage in both the "G1" and "S" phase centrosome preparations. Presence of septin at the centrosome might also be an artefact due to the isolation procedure, since we did not observe any Jurkat or HeLa cells with a centrosomal septin signal by immunofluorescence. No staining was observed when the cells were treated with aphidicolin or submitted to the same osmotic shock as in the centrosome purification procedure (data not shown).

The second protein identified in the same gel band as septin was glutamate dehydrogenase. Since we had no antibodies against this protein, we were unable to verify its centrosomal localisation. Therefore we can not rule out that glutamate dehydrogenase is a contaminant rather than a specific centrosome component.

HCA66, a novel centrosomal protein

We identified HCA66 as a centrosomal protein recruited at the beginning of S phase. The protein was previously identified in patients with hepatocarcinoma (Wang *et al.* 2002). As described in the introduction, defects of the centrosome cycle can lead to genetic instability and cancer. The fact that HCA66 is recruited at the centrosome at the beginning of S phase, at the initiation of the centrosome cycle, and that HCA66 was linked to a carcinoma, make it an attractive candidate that may play a role in centrosome duplication. In HeLa cells, HCA66 is detected at the centrosome from prophase until early anaphase. Why is HCA66 not detected at the centrosome in interphase cells, although the protein is present at "G1" and "S" (and presumably "G2") centrosomes, as shown by our immunofluorescence data on isolated centrosomes? The signal may be too weak compared

to the cytoplasmic background staining, therefore not allowing the detection of the protein at the centrosome. Alternatively, the epitopes might be accessible in the purified centrosomes but not in the cell.

The HCA66 protein is not solely located at the centrosome and has several sub-cellular localisations. HCA66 is also located in the cytoplasm and in the nucleus. It might either reflect a difference of function according to the cellular localisation or the existence of different pools of HCA66, as described for other centrosomal proteins, such as γ -tubulin and centrin (Moudjou *et al.* 1996; Paoletti *et al.* 1996). These different localisations were also described for the 4.1R proteins. Cell fractionation would allow us to determine whether the ratio of cytoplasmic/centrosomal HCA66 undergoes any changes during the cell cycle, such as described for γ -tubulin. Our immunofluorescence data suggest that part of HCA66 shuttles between the chromatin and the spindle pole. At the beginning of mitosis, part of the chromatin-associated HCA66 seems to leave the chromatin and associates with the centrosomes (Figure 18). The association of HCA66 with the chromatin is very peculiar. The protein seems associated with the surface of condensed chromosomes. To test whether additional HCA66 protein was bound to the condensed chromatin but inaccessible to our antibodies, cells were treated with hydrochloric acid after fixation (data not shown). Immunofluorescence on chromosome spreads would allow us to determine whether the protein is associated with the surface of specific chromosomes.

The localisation of HCA66 at the centrosome and in the nucleus reflects a property also found for other proteins. Several other proteins, including NPM/B23, nuclear antigen 14, topoisomerase II α and 4.1R have been described both at the centrosome and in the nucleus (Schmidt-Zachmann *et al.* 1987; Ramos-Morales *et al.* 1998; Okuda *et al.* 2000; Krauss *et al.* 1997a; Krauss *et al.* 1997b; Barthelmes *et al.* 2000; Pfannenschmid *et al.* 2003).

Since there are currently no functional data on HCA66 and no known motifs, suggesting a

function, it is difficult to propose a biological role for this protein. It is conserved from yeast to human, suggesting an important biological function. HCA66 might be involved in cell cycle progression at the G1/S transition, previously shown to be centrosome dependent (Hinchcliffe *et al.* 2001; Khodjakov and Rieder 2001), or in centrosome duplication or formation of the mitotic spindle. More experiments are required to determine the function of HCA66. Depletion of HCA66 by siRNA would enable us to answer some of these questions and understand the role of this protein (Elbashir *et al.* 2001).

Other questions to be addressed include whether HCA66 could act as a licensing factor for centrosome duplication in the same way as NPM/B23. For example, the recruitment of the protein at the beginning of S phase could trigger centrosome duplication. Would over-expression of HCA66 trigger multiple rounds of centrosome duplication? How is the cyclic accumulation of HCA66 to the centrosome regulated? Phosphorylation events triggered by cdk2/cyclin A and E could regulate the recruitment of HCA66 at the centrosome. The use of a specific inhibitor of cdk2/cyclin A and E would help to answer this question.

PCM-1 is recruited to the centrosome in S phase

We showed that the amount of PCM-1 protein was ~1.5 times higher in "S" centrosomes compared to "G1" centrosomes. The recruitment of PCM-1 to the centrosome at the beginning of S phase might reflect its role in recruiting other pericentriolar proteins from the cytoplasm to the centrosome, at the time of centrosome duplication when new PCM is required, as previously proposed (Dammermann and Merdes 2002).

As mentioned in the introduction, centrin and γ -tubulin concentrate at the assembly site of the new centriole. However, we did not detect any changes in the amount of centrin and γ -tubulin between "G1" and "S" centrosomes. This suggests that either cells were arrested before this event happens, or the amount of additional protein recruited is so small that the difference was not detected by immunoblotting or immunofluorescence on isolated centrosomes. Another explanation would be that no additional centrin and γ -tubulin are recruited to the centrosome but that there is a change in the spatial distribution of these proteins within the centrosome. In the case of γ -tubulin, this hypothesis is supported by A. Khodjakov's results who showed that centrosomal γ -tubulin level is constant in interphase and that γ -tubulin is recruited to the centrosome at the onset of mitosis (Khodjakov and Rieder 1999).

4.1R at the centrosome?

The centrosomal localisation of 4.1R protein was first determined by electron microscopy and immunofluorescence in CaSki and WI38 cells (human epithelial cell line and human normal lung fibroblast respectively, Krauss *et al.* 1997b). Other groups also detected the 4.1R protein at the centrosome, using indirect immunofluorescence (Hung *et al.* 2000; Perez-Ferreiro *et al.* 2001a). Hung and collaborators detected 4.1R¹³⁵ at the centrosome of lymphoid T cells, although they failed to detect the protein on purified centrosomes by immunoblotting. None of these studies determined precisely which 4.1R isoforms were located at the centrosome. Moreover, the antibodies used in these previous studies might also detect other 4.1 protein family members which are highly homologous to 4.1R. One of them is 4.1G, which is also known to localise to the centrosome and to the spindle pole (Hoover and Bryant 2000; Delhommeau *et al.* 2002). Finally, more recently, 4.1R was identified as a centrosomal component of KI37 cells (a T lymphoma cell line) by liquid chromatography, followed by mass spectrometry (J. Andersen, personal communication). Thus, it seems established that 4.1R is a centrosomal protein. However, the 4.1R isoforms present at the centrosome have not yet been identified. Since the published literature on the localisation of 4.1R is unclear and sometimes controversial, we decided to investigate this topic.

Using an antibody reacting specifically against 4.1R¹³⁵ and 4.1R⁸⁰ in Jurkat cells, but not against 4.1G (Scott *et al.* 2001), we did not detect any of these isoforms in the centrosome preparation. This result suggests that 4.1R⁸⁰ and 4.1R¹³⁵ are not the 4.1R isoforms present at the centrosome of Jurkat cells, although we can not exclude that these isoforms are present in small quantities below the detection level. Interestingly, 4.1R⁸⁰ and 4.1R¹³⁵ isoforms do not associate with the centrosome but with microtubules when over-expressed in Jurkat

cells, suggesting the absence of these isoforms from the centrosomes of Jurkat cells (Perez-Ferreiro *et al.* 2001a). However, these results have to be taken with caution. The cellular localisation of 4.1R isoforms is very complex. It has been found that an exogenously expressed isoform (4.1R⁸⁰) in COS-7 cells localised in more than one sub-cellular compartment (plasma membrane, nucleus and centrosomes) (Gascard *et al.* 1998). The sorting of the protein to different cellular localisations is likely to be regulated by different post-translational modifications. During our attempts to produce an antibody against 4.1R¹³⁵, the serum 1632 gave a centrosomal staining in all the cell types we tested (HeLa, A6 and Jurkat). Since this antibody was raised against the full-length 4.1R¹³⁵ protein it might react against other isoforms of 4.1R. The antibody recognised a ~210 kDa centrosomal protein which might correspond to the 4.1R isoform located at the centrosome. However, so far the definite proof that this antibody is indeed recognising 4.1R isoforms, and not other 4.1 family members, is missing. A clear answer would be given by immunoprecipitating the 210 kDa protein and identifying it by mass spectrometry.

4.1R and microtubules

Several studies reported an association of 4.1R with microtubules, suggesting a role of 4.1R in maintenance of microtubule architecture (Correas and Avila 1988; Perez-Ferreiro *et al.* 2001a). Both 4.1R¹³⁵ and 4.1R⁸⁰ isoforms can associate with microtubules as demonstrated by immunofluorescence and *in vitro* binding analysis (Perez-Ferreiro *et al.* 2001a). Interestingly, when overexpressed in Jurkat cells, 4.1R isoforms associate with microtubules, whereas ectopic overexpression in COS-7 cells disorganises the microtubule network. Thus, as suggested by other studies (Kontrogianni-Konstantopoulos *et al.* 2000;

Kontrogianni-Konstantopoulos *et al.* 2001), different cell types respond differently to the expression of the same 4.1R isoform, suggesting that functional activities of 4.1R proteins are cell type regulated.

We demonstrated a cross-reactivity of antibodies against epitopes in both 4.1R and β -tubulin. These data indicate a similarity of the 4.1R carboxy-terminus with a short region in β -tubulin, which is further supported by the sequence alignment of the 4.1R C-terminal peptide with the helix 5 region of β -tubulin. Our experiments show that after depolymerisation of microtubules, the 4.1R staining is restricted to the centrosome. This suggests that the antibody can detect both β -tubulin and the centrosomal isoform(s) of 4.1R protein, but under normal conditions, the centrosomal signal is hidden by the high abundance of microtubule filaments in the cytoplasm. We did not detect any plasma membrane staining with this antibody, which might indicate that the epitope is masked in the plasma membrane or that the isoforms recognised by the antibody are not present in the plasma membrane.

What is the biological significance of the similarities between the 4.1R carboxy-terminus and β -tubulin? The localisation of 4.1R protein to the centrosome may suggest a role in microtubule organisation. Microtubules are nucleated from the pericentriolar material, but many of them are subsequently released into the cytoplasm (Keating *et al.* 1997). It is not clear how the anchoring and release of microtubules at the centrosome is regulated. One possibility would be that a protein such as 4.1R is involved in microtubule release. With its carboxy-terminal end showing similarity to β -tubulin, 4.1R could mimic microtubule ends and compete for the binding to centrosomal components, thus disconnecting microtubules from the centrosomal surface. This idea is supported by recent

findings from Perez-Ferreiro and collaborators (Perez-Ferreiro *et al.* 2001a; Perez-Ferreiro *et al.* 2001b). These authors report that over-expression of 4.1R in COS-7 cells disturbed microtubule organisation, and that microtubules no longer radiated from a single pericentriolar focus, although centrosomal markers such as pericentrin or γ -tubulin remained unaltered. Given the multitude of 4.1 isoforms and their varying sub-cellular localisation in different cell types (reviewed in Takakuwa 2000), the 4.1 protein could contribute to the modulation of the microtubule network by releasing microtubules from nucleation sites, and therefore contribute to the various cell shapes and properties of many differentiated cells. The binding of 4.1R to NuMA, as reported by Mattagajasingh and collaborators (Mattagajasingh *et al.* 1999), could provide an additional level of regulation to control microtubule behaviour. Whereas only small amounts of a NuMA splice variant are found at the centrosome during interphase (Tang *et al.* 1994), the majority of NuMA localises near the centrosome in mitosis. In contrast to NuMA, the protein 4.1R appears to be a constitutive resident of the centrosome throughout the cell cycle, binding to the protein CPAP (Hung *et al.* 2000). Therefore, the protein 4.1R could provide a docking site on the mitotic centrosome for NuMA protein complexes that are transported towards the spindle poles by dynein/dynactin (Merdes *et al.* 2000). Similar to interphase cells, the NuMA-4.1R interaction in mitosis might affect processes such as microtubule release from the centrosome (Mastronarde *et al.* 1993).

CONCLUSIONS

Centrosome duplication and the control of the G1/S transition are two important processes whose defect can ultimately lead to cancer. Therefore, the identification of centrosomal proteins involved in these two processes is of primary importance.

In this work, we identified several potential candidates, involved in the centrosome cycle that are differentially localised at "G1" and "S" centrosomes. The characterisation of these proteins and the elucidation of their role at the centrosome will be the next step towards understanding the molecular mechanisms of the centrosome cycle.

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Appendix 1: Sequences

Sequence of HCA66 cDNA (accession number: NM_018428) with the ATG and stop codon indicated in red. 5' and 3' untranslated regions (UTR) are in blue.

GCTGTTGAGAAGCTACCCGCGGGTGTAGACCTCGGACCTCATGGCAGAGATAATTCAGGA
ACGCATAGAAGATCGGCTCCCGGAATTGGAACAGCTGGAGCGCATTTGGACTGTTCAAGTCATG
CGGAGATTAAGGCTATCATTAAGAAGGCTTCCGATCTAGAGTACAAAATCCAGAGAAGAACC
CTTTTCAAGGAAGACTTTATCAATTATGTTCAATATGAAATTAATCTTTTGGAGCTGATCCG
GAGAAGAAGAACACGCATTGGATATTCATTTAAGAAGGATGAGATTGAGAATTCTATTGTAC
ACCGGGTACAAGGTGTTTTCCAGCGTGCCTCAGCAAAATGGAAAGACGATGTTCAACTTTGG
CTCTCCTATGTGGCTTTTTGTAAGAAGTGGGCTACTAAAACCTCGACTTAGCAAGGTATTCTC
TGCCATGTTGGCGATTCAATCCAACAAACCAGCTTTGTGGATTATGGCAGCCAAATGGGAAA
TGGAAGATCGATTGTCTTCAGAAAGCGCAAGGCAACTATTTCTTCGCGCACTGCGCTTTCAT
CCAGAGTGCCCCAAAACCTTTATAAAGAATACTTTAGGATGGAGCTGATGCATGCTGAAAAACT
GAGGAAGGAGAAGGAAGAATTTGAAAAAGCCAGTATGGATGTGGAGAATCCTGATTATTCTG
AAGAAATCCTTAAGGGCGAGTTGGCATGGATCATCTACAAAATTTCTGTAAGCATAATTAAA
GGTGCAGAATTTACGTGTCACTGCTTTTCGATTGCACAGCTATTTGACTTTGCCAAAGATCT
ACAAAAAGAGATTTATGATGACCTTCAGGCTCTACACACAGATGATCCTCTCACTTGGGATT
ATGTGGCAAGGCGAGAATTAGAGATTGAGTCACAGACAGAAGAGCAGCCTACAACGAAACAA
GCCAAAGCAGTGGAGGTCGGCCGGAAGGAGGAGAGGTGCTGTGCTGTGTATGAAGAGGCAGT
GAAGACTCTGCCAACAGAGGCCATGTGGAAGTGTTACATCACCTTTTGCTTGGAAGAGTTTA
CTAAGAAGTCAAATAGTGGGTTTCCTTAGAGGGAAGAGGTGGAAGAACCATGACTGTATTC
AGGAAGGCACATGAACTGAAGCTTCTGTCAGAATGCCAATACAAGCAGTTGAGTGTTTCGTT

GCTGTGTTATAACTTCCTGAGGGAAGCTCTGGAAGTGGCAGTAGCTGGAAGTGAATTGTTTA
GAGACTCTGGGACAATGTGGCAGCTGAAGCTGCAGGTGCTGATCGAGTCAAAGAGCCCTGAC
ATAGCCATGCTTTTTGAAGAAGCCTTTGTGCACCTGAAACCCAGGTTTGTCTGCCATTGTG
GATTTCTTGGGCAGAGTGGAGTGAAGGTGCCAAAAGCCAAGAAGACACTGAGGCAGTCTTTA
AGAAAGCTCTCTTAGCTGTCATAGGTGCCGACTCAGTAACCCTGAAGAATAAGTACCTGGAT
TGGGCTTATCGAAGTGGTGGCTACAAAAAGGCCAGAGCTGTGTTTAAAAGTTTACAGGAGAG
CCGACCATTTTCAGTTGACTTTTTTCAGGAAAATGATTCAGTTTGAAAAGGAGCAAGAATCCT
GCAATATGGCGAACATAAGAGAATATTATGAGAGAGCTTTGAGAGAGTTTGGATCCGCAGAT
TCTGATCTTTGGATGGATTATATGAAAGAAGAATTGAACCACCCCCTTGGTAGACCTGAGAA
CTGTGGACAGATCTACTGGCGAGCGATGAAAATGTTGCAGGGAGAGTCAGCAGAGGCATTTG
TAGCTAAACATGCTATGCATCAGACTGGCCATTTATGAAGATGAAGAATACCGTCCGCTTTG
TGAAATAGTATTGCAAGCCAGCCCCGTGGGCAAATTTGTATTGAGTCCATCTGTAATTTGCT
CAGTGATGGCAGACAAGATGGCTGTCTGGTTTTGAGACACACACTTTAATTTTATGTAACT
TGTTAAATCTTTTTTAAAAATTAAAAAATTTTTATGATTGAAAAAAAAA

HCA66 protein sequence (accession number: NP_060898)

MAEIIQERIEDRLPELEQLERIGLFSHAEIKAIKKASDLEYKIQRRTLFKEDFINYVQYEI
NLLELIRRRRTRIGYSFKKDEIENSIVHRVQGVFQRASAKWKDDVQLWLSYVAFCKKWATKT
RLSKVFSAMLAIHSNKPALWIMAAKWEMEDRLSSESARQLFLRALRFHPEC PKLYKEYFRME
LMHAEKLRKEKEEFKASMDVENPDYSEEILKGELAWIIYKNSVSI IKGAEFHVSLLSIAQL
FDFAKDLQKEIYDDLQALHTDDPLTWDYVARRELEIESQTEEQPTTKQAKAVEVGRKEERCC
AVYEEAVKTLPTTEAMWKCYITFCLE RFTKKSNSGFLRGKRLERTMTVFRKAHELKLLSECQY
KQLSVSLLCYNFLREALEVAVAGTELF RD SGTMWQLKLQVLIESKSPDIAMLFEEAFVHLKP
QVCLPLWISWAEWSEGAKSQEDTEAVFKKALLAVIGADSVTLKNKYLDWAYRSGGYKKARAV
FKSLQESRPFVDFFRKMIQFEKEQESCNMANIREYYERALREFGSADSDLWMDYMKEELNH
PLGRPENCGQIYW RAMKMLQGESA EAFVAKHA MHQTGHL

cDNA encoding *Xenopus laevis* 4.1R¹³⁵ sequence (accession number: M20621) with the ATG and stop codon indicated in red. 5' and 3' untranslated regions (UTR) are in blue.

CGGGATTTGGGCGCGTCACGTGACGGGAGTTGAAGCGCGGGAAGCTGGTAGCCGACCCGAAA
TACGGTAACAATGACTACAGAGAAGGGGTATTAGCTGAAGCAGAAAGCCCTCCTCAGGACC
AAAAGCAAGAAGGAGAGGAGGAAGTAGAATCTTGTACAACCTCAACCAGTTGTTGGCAGTGGT
GATAAGGATCCAGAGACTGAACAGTCACAAGAGTCACCTTCCACTACCTCTCCAAGCACCCG
CAAGAGCAAGGACCGACATTACAGGGAAAGGGCCTCTCTCGCCTCTTTTCCTCTTTCCTGA
AGAGACCCAAGTCCCAGGTGTCTTCTGATGAAAAGGAAGTTGAACTGTTGGGGGAAAAGGGA
CAAGACCAAAAAGGATGTTGATGAGGGTTTGGGAGAGCAGCTAGAGGATGATGTTTTTCTAAA
GGCCCCCTATTGCAGCTCCAGAGCCTGAACTTCGAACTGACCCATCACTAGACCTTCATTCTCT
TAAGTAGTGCAGAAACCCAGCCAGCACAAAGAGGAACAGAAAGAAGACCAAGACCCAGAAGCA
GACTGTGAGGACGTGGAAGGCAAGGAACCAATAAAAAAACCTGAAGGAGAATCAAAGGCCTC
GCATAAAGTAGTTAGGCGCTCTCCCAACATGCGCTGCAAAGTCACACTTCTCGATGACACTG
TTTATGAGTGTGACTTGGAGAAACATGCAAAGGGACAGGACATATTTAAGAAAGTCTGCAGC
CACCTTAATATCGTGGAGGAAGATTATTTTGGATTGGCAATATGGGAATCTCCAACCTTGCAA
GGTCTGGCTTGATCCTTTGAAGGACATCAGGAAGCAGGTTTCATGGAGGTCCATGTGAATTCA
CTTCCAATGTGAAGTTTTATCCCCCTGATCCGGCACAGTTGTCAGAGGATATAACCAGGTAC
TACCTGTGTCTCCAGCTGCGCAAAGATATTTTCAGTGGGCGTCTTCCATGCTCATTTGCCAC
CCTTGCTCTGCTCGGTTCTTATACTGTGCAATCAGAGGTGGGTGACTATGAAGAGGATCTCC
ATGGTGTAGACTATGTCAGTGAATTTAAACTCAGTCCAAATCAAACAAAAGATCTTGAAGAG
AAGGTTGGGGAGCTTCATAAGTCATATAGATCTATGACTCCTGCTCAGGCTGACCTGGAATT
CCTGGAGAATGCCAAAAAGCTTACCATGTATGGTGTAGATATTCACCAAGCCAAGGACTTGG
AGGGAGTGGATATCAAACCTTGGGGTTTGCTCTGGTGGTCTTATGGTTTTCAAAGACAATCTG
AGGATCAACCGCTTTCATGGCCCAAAGTACTGAAAATCTCCTATAAAAGAAGTAGTTTCTT
CATCAAAATCCGCCCTGGAGAGCAGGAGCAATATGAAAGTACAATAGGATTTAAGCTTCCCA
GTTACAAAGCAGCAAAGAACTTTGGAAAGTATGTGTTGAACATCATACATTTTTTCAGGTTG
ACATCCACAGAGTCTATTCCCAAACACAGATTTCTTTCTCTAGGATCCACATTTTCGCTACAG

TGGGCGCACACAGGCACAGACTCGACATGCCAGTGCTCTTATTGATCGACCTGCTCCCCATT
TTGTACGCACTGGCAGCAAACGGGCCTCTCGCAGTCTTGATGGAGCTGCAGTTGCAACCCCA
GAAGCCTCCAGGACCCACCGCCCTGTATCAGCCCCGGTCTTCCCACCGGAATTCCCTGCGGT
CCAAAGAAAGACTCCAGGGCCAAGAGTAGAGGAAATGCCCAAAAAGACAGAGGAGAAGCCCA
AAGAAGGGATGCCAAATCAGCGGGAAAGTCCTAAGGATGTTAAAGCTACACAGCAAGATTCT
CCTTCACCTACTGTCAATGGGGATAAAGTTAAGGACCTGGAAAAGACACAGGACGAGATCAT
CAGACACCATGCTAGCATCCGTGAGTTGAAGAAGAGTTTCATGGAGTCTGTGCCAGCACCCC
GGCCCAGCGAATGGGACAAACGACTTTCTACCCACTCCCCTTTCCGTACTCTCAGCTTTAAT
GGCCAGGTCCAGACTGGGACAGATGGGCCTCCACTAGTGAAAACACAGACCGTCACCATCTC
CAATGCAACAAACGGTGAAAAGGGAGAGATTCCAATAAGGAGGTTCCCTTGGTACACACTG
AGACCAAAACAATCACATACGAAGCAGCCAGATCTGATGATGTTAACTGACCAGGAACCT
GGCATTCTACTAACCGCTCATACAATCACTTCTGAGACCACAAGTAGTACCACCACCACCCA
GATCACAAGACTGTAAAAGGTGGCATCTCAGAGACACTCATAGAGAAAAGGATAGTCATCA
CAGGAGATGGAGACCTTGATCATGACCAGGTCTTAGTTCAAGCTATTAAAGAAGCAAAGGAG
CAGACCCAGACATGTCTGTTACCAAGGGTGTTGTTTCATCAAGAGACAGAAATAGCTTAAGA
ATACCAGGAGGCGGCACAGACTTTTAACAGGACACATTAAAGACTTTCCAGAAAACCAGCAG
TTCCCTGAAGTATCCTCCCTCACCCCTGGGACATTGTCAGCAGCCACTGGAATTTTCTCTCT
CTGTTTTACCTCAACCACAACAATCCACATTTCTGTGTTTGATCAAAGACTGTCTCCCCCT
CGAACATGGAATTTACAATGTGACGCCACAACCTGTAACTCCTTCAAAACTAATGTTGAAA
TGAAATGGCTTCCCATATCAGAATGAATTC

Protein sequence of *Xenopus laevis* 4.1R¹³⁵ (accession number: AAA49695)

MTTEKGLLAEAESPPQDQKQEGEEVESCTTQPVVGS GDKDPETEQSQESPSTTSPSTRKSK
DRHSQGKGLSRLFSSFLKRPKSQVSSDEKEVELLGEKGGDQKDVDEGLGEQLEDDVFLKAPI
AAPEPELRTDPSLDLHSLSSAETQPAQEEQKEDQDPEADCEDVEGKEPIKKPEGESKASHKV
VRRSPNMRCKVTLLDDTVYECDLEKHAKGQDIFKKVCSHLNIVEEDYFGLAIWESPTCKVWL
DPLKDIRKQVHGGPCEFTSNVKFYPPDPAQLSEDI TRYYLCLQLRKDIFSGRLPCSFATLAL
LGSYTVQSEVG DYEDLHGVDYVSEFKLSPNQTKDLEEKVGELHKSYSRSMTPAQADLEFLEN
AKKLTMYGVDIHQAKDLEGVDIKLGVC SGGLMVFKDNL RINRFPWPKVLKISYKRSSFFIKI
RPGEQE QYESTIGFKLPSYKAAKKLWKVCVEHHTFFRLTSTESIPKHRFLSLGSTFRYSGRT
QAQTRHASALIDRPAPHFVRTGSKRASRLDGAAVATPEASRTHRPVSAPVFPPEFPAVQRK
TPGPRVEEMP KKTEEKPKEGMPNQRESPKDV KATQQDSPSPTVNGDKVKDLEKTQDEIIRHH
ASIRELKKSFMESVPAPRPSEWDKRLSTHSPFRTL SFNGQVQTGTDGPPLVKTQTVTISNAT
NGEKGEIPTKEVPLVHTETKTITYEAARSDDVNTDQEPGILLTAHTITSETTSSTTTTQITK
TVKGGISETLIEKRIVITGDGDL DHDQVLVQAIKEAKEQHPDMSVTKGVVHQETEIA

cDNA sequence of MCC1 (accession number: M62397) with the ATG and stop codon indicated in red. 5' and 3' untranslated regions (UTR) are in blue.

GGCACGAGGGAAACTTCGGCGTGAAGTGCAGCTCCGCTCCGGCTCCCTCTAGCTTTCTTTCC
TTCTCTGGAATCCGAGGCGCGGATCTTCCTCGCCCCACCGCCCTAGTTTTTTTCGGGAGCTCG
CCGGTGCCCTCTAGGGTGTTCGGCTCGTGCTGGGAAGTGCCCTCCATCCTGGTAATGGGGGGC
GGCGAGGCACCGTAGGAGTGGCGAGGCGGCGCCCAGGGTGGCACTGCCCCGGAACGGGGCGC
TGGGTGCGCGCGGGAGGGTCCCCGCGCGGGCTCCGCCGCTGCCGCAGCTGCGAGCGCGCCGC
GCCACCGAGCCTCCTGCAGCAATGGCTCGTCCGTGAAACGCGAGCCACGGCTGCTCTTTTAA
AGAGTGCCTGCATCCTCCGTTTGCGCTTCGCAACTGTCTGGGTGAAAATGGCTGTCTAGAC
TAAAATGTGGCAGAAGGGACCAAGCAGTGGATATTGAGCCTGTGAAGTCCAACTCTTAAGCT
CCGAGACCTGGGGGACTGAGAGCCCAGCTCTGAAAAGTGCATCATGAATTCCGGAGTTGCCA
TGAAATATGGAAACGACTCCTCGGCCGAGCTGAGTGAGCTCCATTACAGCAGCCCTGGCATCA
CTAAAGGGAGATATAGTGGAACCTAATAAACGTCTCCAGCAAACAGAGAGGGAACGGGACCT
TCTGGAAAAGAAATTGGCCAAGGCACAGTGCGAGCAGTCCCACCTCATGAGAGAGCATGAGG
ATGTCCAGGAGCGAACGACGCTTCGCTATGAGGAACGCATCACAGAGCTCCACAGCGTCATT
GCGGAGCTCAACAAGAAGATAGACCGTCTGCAAGGCACCACCATCAGGGAGGAAGATGAGTA
CTCAGAACTGCGATCAGAACTCAGCCAGAGCCAACACGAGGTCAACGAGGACTCTCGAAGCA
TGGACCAAGACCAGACCTCTGTCTCTATCCCCGAAAACCAGTCTACCATGGTTACTGCTGAC
ATGGACAACTGCAGTGACCTGAACTCAGAACTGCAGAGGGTGCTGACAGGGCTGGAGAATGT
TGTCTGCGGCAGGAAGAAGAGCAGCTGCAGCCTCTCCGTGGCCGAGGTGGACAGGCACATTG
AGCAGCTCACACAGCCAGCGAGCACTGTGACCTGGCTATTAAGACAGTCGAGGAGATTGAG
GGGGTGCTTGGCCGGGACCTGTATCCCAACCTGGCTGAAGAGAGGTCTCGGTGGGAGAAGGA
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AACTGAACCGGACTAAGGCCACCATGAATGCCATCCGGGAAGAGCGGGACCGGCTCCGGAGG
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GCAGCAATGACATTCCCATCGCCAAGATTGCTGAGAGGGTGAAGCTATCAAAGACAAGGTCC

GAATCGTCATCATCTGATCGGCCAGTCCTGGGCTCAGAAATCAGTAGCATAGGGGTATCCAG
CAGTGTGGCTGAACACCTGGCCCACTCACTTCAGGACTGCTCCAATATCCAAGAGATTTTCC
AAACACTCTACTCACACGGATCTGCCATCTCAGAAAGCAAGATTAGAGAGTTTGAGGTGGAA
ACAGAACGGCTGAATAGCCGGATTGAGCACCTCAAATCCCCAAAATGACCTCCTGACCATAAC
CTTGAGGAATGTAAAAGCAATGCCGAGAGGATGAGCATGCTGGTGGGAAAATACGAATCCA
ATGCCACAGCGCTGAGGCTGGCCTTGACAGTACAGCGAGCAGTGTCATCGAAGCCTACGAATC
CTCCTGGCGCTGGCAGAGAGTGAGCAGAGCCTCATCCTGGGGCAGTTCCGAGCGGCGGGCGT
GGGGTCCTCCCCTGGAGACCAGTCGGGGGATGAAAACATCACTCAGATGCTCAAGCGAGCTC
ATGACTGCCGGAAGACAGCTGAGAATGCTGCCAAGGCCCTGCTCATGAAGCTGGACGGCAGC
TGTGGGGGAGCCTTTGCCGTGGCCGGCTGCAGCGTGCAGCCCTGGGAGAGCCTTTCCTCCAA
CAGCCACACCAGCACAAACCAGCTCCACAGCCAGTAGTTGCGACACCGAGTTCATAAAGAAG
ACGAGCAGAGGCTGAAGGATTATATCCAGCAGCTCAAGAATGACAGGGCTGCGGTCAAGCTG
ACCATGCTGGAGCTGGAAAGCATCCACATCGATCCTCTCAGCTATGACGTCAAGCCTCGGGG
AGACAGCCAGAGGCTGGATCTGGAAAACGCAGTGCTTATGCAGGAGCTCATGGCCATGAAGG
AGGAGATGGCCGAGTTGAAGGCCCAGCTCTACCTACTGGAGAAAGAGAAGAAGGCCCTGGAG
CTGAAGCTGAGCACGCGGGAGGCCCAGGAGCAGGCCTACCTGGTGCACATTGAGCACCTGAA
GTCCGAGGTGGAGGAGCAGAAGGAGCAGCGGATGCGATCCCTCAGCTCCACCAGCAGCGGCA
GCAAAGATAAACCTGGCAAGGAGTGTGCTGATGCTGCCTCCCCAGCTCTGTCCCTAGCTGAA
CTCAGGACAACGTGCAGCGAGAATGAGCTGGCTGCGGAGTTCACCAACGCCATTCGTGCGAGA
AAAGAAGTTGAAGGCCAGAGTTCAAGAGCTGGTGAGTGCCTTGGAGAGACTCACCAAGAGCA
GTGAAATCCGACATCAGCAATCTGCAGAGTTCGTGAATGATCTAAAGCGGGCCAACAGCAAC
CTGGTGGCTGCCTATGAGAAAGCAAAGAAAAAGCATCAAAACAACTGAAGAAGTTAGAGTC
GCAGATGATGGCCATGGTGGAGAGACATGAGACCCAAGTGAGGATGCTCAAGCAAAGAATAG
CTCTGCTAGAGGAGGAGAACTCCAGGCCACACACCAATGAACTTCGCTT**TAATCAGCACTC**
ACGCACCGGAGTTCTGCCCATGGGAAGTAACTGCAGCAGGCCACTGGGGACAGAAGGGCCC
ATGTACTTGTTGGGAGGAGGAGGAAAGGGAAGGCTGGCAGGTAGGTGGCACTTGGACAATG
GAGTGCCCCAACTCAACCCTTGGGGTGA**CTGGCCATGGTGACATTGTGGACTGTATCCAGAG**
GTGCCCGCTCTTCCCTCCTGGGCCCAACAGCGTGTA**AACACATGTTCTGTGCCTGCTCAG**

CAGAGCCTCGTTTCTGCTTTCAGCACTCACTCTCCCCCTCCTCTTCTGGTCTGGCGGCTGTG
CATCAGTGGGATCCCAGACATTTGTTTCTGTAAGATTTCCATTGTATCCTCTTTTTGGTAG
ATGCTGGGCTCATCTTCTAAAAAAAAAAAA

Protein sequence of MCC1 (accession number: AAA52069)

MNSGVAMKYGNDSSAELSELHSAALASLKGDIVELNKRLQQTERERDLLEKKLAKAQCEQSH
LMREHEDVQERTTLRYEERITELHSVIAELNKKIDRLQGTTIREEDEYSELRSELSQSQHEV
NEDSRSMDDQTSVSI PENQSTMVTADMDNCSDLNSELQRVLTGLENVVCGRKKSSCSLSVA
EVD RHIEQLTTASEHCDLAIKTVEEIEGVLGRDLYPNLAEERSRWEKELAGLREENESLTAM
LCSKEEELNRTKATMNAIREERDLRRRVRELQTRLQSVQATGPSSPGRLTSTNRPINPSTG
ELSTSSSSNDIPIAKIAERVKLSKTRSESSSSDRPVLGSEISSIGVSSSVAEHLAHS LQDCS
NIQEIFQTLYSHGSAISESKIREFEVETERLNSRIEHLKSQNDLLTITL EECKSNAERMSML
VGKYESNATALRLALQYSEQCIEAYELLLALAESEQSLILGQFRAAGVGSSPGDQSGDENIT
QMLKRAHDCRKTAENAAKALLMKLDGSCGGAFAVAGCSVQPWESLSSNSHTSTTSSTASSCD
TEFTKEDEQRLKDYIQQLKNDRAAVKLTMLELESIHIDPLSYDVKPRGDSQRLDLENVLMQ
ELMAMKEEMAELKAQLYLLEKEKKALELKLSTREAQEAYLVHIEHLKSEVEEQKEQRM RSL
SSTSSGSKDKPGKECADAASPALSLAELRTTCSENELAAEFTNAIRREKKLKARVQELVSAL
ERLTKSSEIRHQQSAEFVNDLKRANSNLVAAYEKAKKKHQNK LKKLESQMMAMVERHETQVR
MLKQRIALLEEENS RPHTNETSL

Appendix 2: Publication

Fant X, and Merdes A (2002). The carboxy-terminus of protein 4.1R resembles β -tubulin.
Cell Biol. Int. 26: 371-377.



THE CARBOXY-TERMINUS OF PROTEIN 4.1R RESEMBLES BETA-TUBULIN

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The protein 4.1R is an isoform of a larger family of 4.1 proteins. It is known as a component of the plasma membrane skeleton, but it is also found at the centrosomes in interphase and mitosis. To investigate the properties of the carboxy terminal region of protein 4.1R, we raised antibodies against a peptide representing the last 14 amino acids of 4.1R. These antibodies crossreact with an epitope in beta-tubulin and stain the microtubule network by immunofluorescence. Furthermore, sequence comparison of the carboxy terminal 4.1R peptide sequence with tubulin reveals homology with a region at the end of helix 5 in beta-tubulin, but not alpha-tubulin. A potential function of the 4.1R carboxy terminus in regulating the formation of microtubule networks is discussed.

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KEYWORDS: microtubules; NuMA; centrosome; mitosis.

INTRODUCTION

The protein band 4.1 has been identified about thirty years ago as a major constituent of the membrane skeleton in erythrocytes (Fairbanks *et al.*, 1971). It has been named according to its electrophoretic mobility on protein gels of erythrocyte ghosts, and it has been characterized as a binding partner of other membrane skeleton elements, including spectrin and actin. Closely related isoforms of 4.1 exist in other cell types, encoded by at least four different genes in humans: 4.1R in red blood cells and most other tissues, 4.1N in central and peripheral neurons, 4.1B in brain, and 4.1G which is generally expressed throughout the body (for an overview, see Hoover and Bryant, 2000). Further variability is generated by alternative splicing; e.g. the gene coding for 4.1R contains 21 exons which can be arranged in a multitude of splice variants (Takakuwa, 2000).

In a search for interactors of 4.1R, components of the mitotic spindle pole including NuMA (protein of the nucleus and mitotic apparatus, Mattagajasingh *et al.*, 1999) and the novel centrosomal protein CPAP (centrosomal protein 4.1-associated protein, Hung *et al.*, 2000) have been

identified. These findings were further supported by localization studies, demonstrating that a significant amount of 4.1R localizes to centrosomes both during interphase and mitosis (Krauss *et al.*, 1997). The exact role of protein 4.1R on the centrosome is not clear. Mattagajasingh *et al.* (1999) suggested that 4.1R may be crucial for the organization of the mitotic spindle. In support of this, Clark and Meyer (1999) suggested that 4.1R might be part of a complex that binds to NuMA and the actin-related protein Arp1, an interaction that has previously been shown to be important for spindle formation (Merdes *et al.*, 1996, 2000).

To investigate the role of 4.1R in the mitotic spindle in more detail, we raised antibodies against a peptide sequence in the C-terminal domain of this protein. In this report, we demonstrate that antibodies against this region cross-react with beta-tubulin and that the 4.1R carboxy terminus shares sequence similarities with a region in beta-tubulin oriented towards the outer surface of polymerized microtubules.

MATERIALS AND METHODS

Generation of antibodies against 4.1R

A peptide containing the carboxy-terminal amino acid sequence SVTKGVVHQETEIA of 4.1R in

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Xenopus laevis was synthesized by Sigma/Genosys (Cambridge, England), including an additional amino-terminal cysteine residue for coupling of the peptide to keyhole limpet hemocyanine. The coupled peptide was injected into two rabbits for immunization, followed by five additional injections to boost the immune response. Serum was collected on day 77 after immunization. The serum was affinity purified either over a tubulin affinity column, made of phosphocellulose-purified bovine brain tubulin, coupled to CNBr-activated Sepharose 4 Fast Flow (Amersham Pharmacia, Uppsala, Sweden), or over nitrocellulose western blot strips containing a single protein band of the full-length recombinant 4.1R protein.

The recombinant 4.1R protein was generated in bacteria; for this purpose the cDNA of clone pXFPO/SP64T encoding full-length *Xenopus* 4.1R (a gift from Dr Randall Moon, University of Washington, Seattle, U.S.A.; Spencer *et al.*, 1990) was PCR-amplified using Pfu polymerase (Stratagene, La Jolla, California, U.S.A.) and primers at the extreme end of the 4.1R coding region, and cloned into the BamHI site of pRSET-B (Invitrogen, Groningen, The Netherlands) after blunting with Klenow fragment. The integrity of the sequence was verified by sequencing. The cloning in pRSET-B added an amino-terminal hexa-histidine tag to 4.1R that was used to purify the protein over nickel-sepharose.

Antibodies were tested by probing dot blots of the 4.1R peptide or a control peptide of the tail domain of *Xenopus* NuMA (EPKELEELREKNESL), using 6 µg peptide/well. They were further tested by immunoblotting of cell extracts, purified 4.1R, or tubulin on nitrocellulose. Cell extracts were made from cultured *Xenopus* A6 kidney cells, homogenized and boiled in protein gel loading buffer containing sodium dodecyl sulfate, or from *Xenopus laevis* eggs, as described by Murray (1991).

A different antibody was raised in a mouse, immunized with a hexa-histidine tagged 4.1R fusion protein containing the carboxy-terminal 271 amino acids of *Xenopus* 4.1R. This fusion protein was obtained by PstI digest of pXFPO/SP64T, cloning of the resulting 1100 bp fragment into pRSET-B, expression in *E. coli* BL-21, and purification over nickel-sepharose.

Cloning and expression of recombinant alpha and beta-tubulin

Clones for alpha and beta-tubulin cDNA were provided by Dr. Don Cleveland (La Jolla,

California, U.S.A.; see Cowan *et al.*, 1983; Lopata *et al.*, 1983). The original cDNAs in pBR322 were amplified by PCR using primers at the extreme 5' and 3' ends of the coding region, and cloned into pGEX 4T2 (Amersham Pharmacia, Uppsala, Sweden). The pGEX 4T2 vector added an amino-terminal glutathione-S-transferase tag that was used for subsequent purification of the bacterially expressed fusion proteins over glutathione affinity columns.

Cell culture and microscopy

HeLa cells, chicken DU249 cells, and *Xenopus* A6 kidney cells were cultured in DMEM, RPMI 1640, or 66% strength L-15 medium, respectively. All culture media were supplemented with 10% fetal calf serum, L-glutamine, penicillin and streptomycin (all reagents from Gibco BRL, Paisley, Scotland). Cells were grown on glass coverslips and fixed in methanol at -20°C for 10 min, re-hydrated in phosphate-buffered saline, and labelled with antibodies against tubulin (DM1 alpha, Sigma, St Louis, Missouri, U.S.A.) or protein 4.1. As secondary antibodies, Texas Red-coupled horse anti-mouse (Vector Laboratories, Burlingame, California, U.S.A.) and FITC-coupled donkey anti-rabbit were used. DNA was stained using 4',6'-diamidino-2-phenylindole (DAPI). In some samples, microtubules were depolymerized with 25 µM nocodazole for 45 min at 4°C . Samples were viewed and documented with a Zeiss Axioskop 2 and a Zeiss Axiocam digital camera, using software from the manufacturer (Carl Zeiss, Oberkochen, Germany).

RESULTS

A polyclonal antibody against the carboxy-terminal 271 amino acids of 4.1R was raised in a mouse, showing the typical plasma membrane skeleton staining along cell-cell contacts in cultured cells (Fig. 1) that has been documented previously in the literature (Lue *et al.*, 1994). Besides, a clear staining of the centrosomal region is visible, both in interphase and mitotic cells. This finding is consistent with earlier work by Krauss *et al.* (1997). Because 4.1R has been reported to interact with NuMA, and because isoforms of NuMA were also found at the centrosome in interphase and mitosis (Tang *et al.*, 1994), we attempted to characterize this interaction in more detail by raising new antibodies, directed specifically against the NuMA binding site of 4.1R.

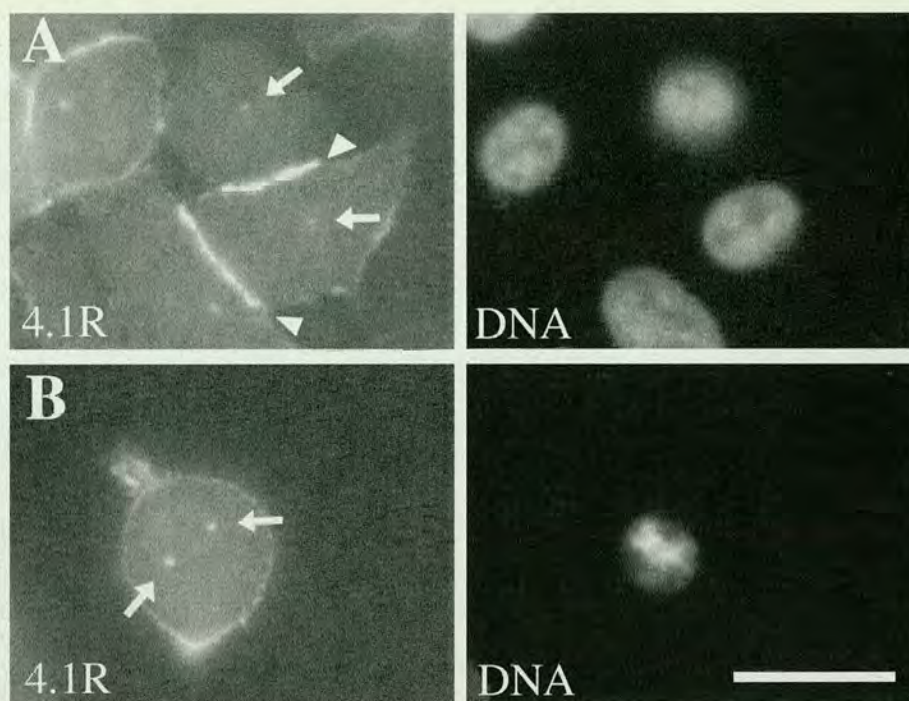


Fig. 1. Polyclonal antibodies against 4.1R stain the plasma membrane at the sites of cell-cell contact, and the centrosomes during interphase and mitosis. Chicken hepatoma DU249 cells were stained with mouse anti-4.1R serum in interphase (A) and mitosis (B). The corresponding DNA staining with DAPI is shown on the right. Arrows indicate centrosome staining, arrowheads indicate the plasma membranes at cell-cell contact sites. Bar in B (right), 20 μ m.

This binding site was mapped to exons 20 and 21 of 4.1R (Mattagajasingh *et al.*, 1999), represented by the carboxy-terminal 60 amino acids of our *Xenopus* 4.1R isoform. Because of the high conservation of the last 14 amino acids within this region (93% identity between human and *Xenopus* 4.1R), we assumed a functional importance and raised antibodies against a synthetic peptide of this sequence. Two rabbits were immunized and gave positive immune responses to the 4.1R peptide, as seen on dot blots (Fig. 2). Both sera showed only background reactivity against a control peptide from *Xenopus* NuMA (Fig. 2).

The sera were further tested by immunoblotting, and both gave identical results. For this reason, only serum 1 is described in the following: Whereas the preimmune serum did not contain any recognizable titer, the immune serum showed a strong reaction against recombinant 4.1R, containing the sequence of the immunizing peptide at its carboxy-terminal end. In whole cell extracts of *Xenopus* A6 kidney cells, however, the serum predominantly reacted against a protein band at 55 kDa, suggesting tubulin. In a separate lane, loading of pure brain tubulin indicated that this 55 kDa band represents tubulin. Furthermore, immunofluorescence labelling of cultured HeLa cells showed a

filamentous staining pattern reminiscent of microtubules (Fig. 3A,D). Double immunofluorescence with a specific antibody against tubulin confirmed that the peptide antibodies against 4.1R stained indeed the microtubule network (Fig. 3B, C, E, F). This staining could be completely eliminated by treatment of the cells with nocodazole: Fig. 3G and H show that the microtubule network was completely depolymerized, and that the staining with 4.1R antiserum was now restricted to the area of the centrosomes, whereas pre-immune serum failed to detect any centrosomal signal (Fig. 3I, J).

Because identical properties were observed in the immune sera from both rabbits, we believed that the detection of tubulin by immunoblotting and immunofluorescence was due to a crossreactivity with the 4.1R epitope, rather than due to an independent antibody titer against tubulin, as sometimes accidentally appearing in a rabbit serum. However, to distinguish between these two possibilities, we affinity purified serum from recombinant 4.1R protein. As shown by immunoblotting and immunofluorescence (Fig. 4A, B), the reactivity against tubulin was still preserved in the anti-4.1R antibody, indicating that the same antibody crossreacted with both 4.1R and tubulin. Consistently, serum that was affinity purified from

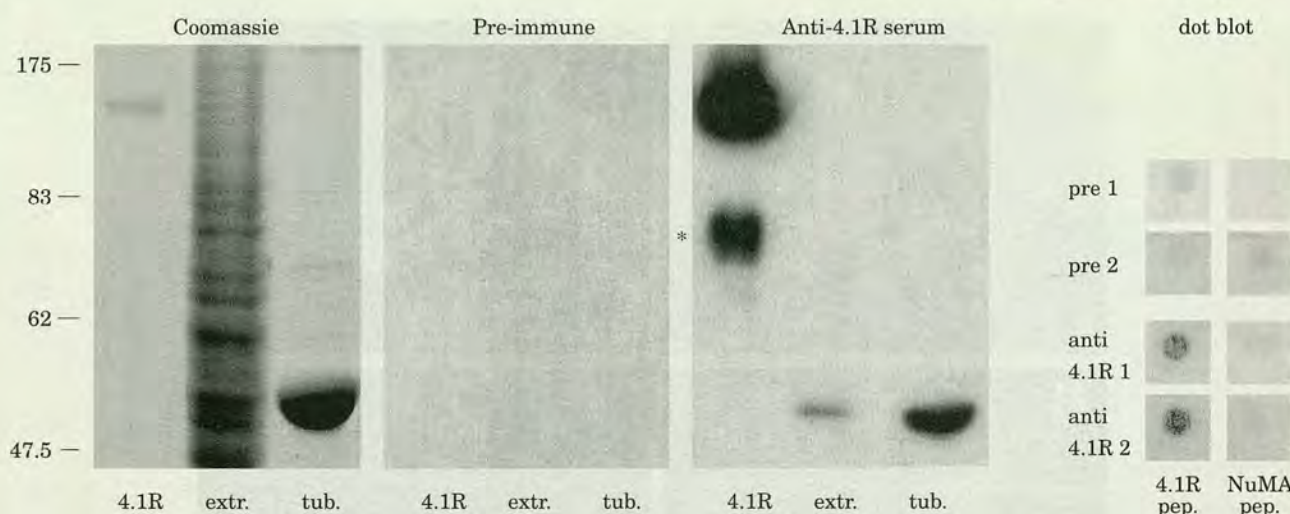


Fig. 2. Serum against a 4.1R C-terminal peptide recognizes tubulin. Protein gels containing recombinant full-length 4.1R (lane 4.1R), extract of *Xenopus* A6 cells (lane extr.), and bovine brain tubulin (lane tub.) were stained with Coomassie, or blotted onto nitrocellulose and probed with rabbit pre-immune serum, or serum after immunization with a peptide representing the C-terminal 14 amino acids of *Xenopus* 4.1R. The asterisk on the anti-4.1R immunoblot indicates a degradation product of the recombinant 4.1R protein that reacts strongly with our serum. On the right, dot blots of the 4.1R C-terminal peptide and a control peptide of the tail domain of *Xenopus* NuMA are shown. These were probed with pre-immune sera, as well as immune sera from two different rabbits.

phosphocellulose-purified brain tubulin also recognized 4.1R protein by immunoblotting (Fig. 4A, C). To our surprise, the antibodies only recognized the beta-tubulin isoform, but not alpha-tubulin. This was verified by immunoblotting against recombinant fusion proteins, containing alpha or beta-tubulin tagged with glutathione-S-transferase (Fig. 4A).

Finally, an alignment of the 4.1R peptide sequence with alpha and beta-tubulin using Clustal W software (EBI, Hinxton, U.K.) revealed 29% identity with amino acids 183 to 196 in beta-tubulin, but no significant similarity in alpha-tubulin (Fig. 5). The specified region is located at the end of helix 5 in beta-tubulin, and is exposed to the outer surface of the microtubule polymer, according to the structural analysis by Nogales *et al.* (1998, 1999). Although the degree of similarity between the 4.1R peptide and beta-tubulin is weak and only restricted to a very short region, the specificity of the antibodies for beta-tubulin and the accessibility of the epitope for immunofluorescence labelling support the idea that the antibody crossreactivity is directed against this area of the tubulin molecule. Conventional BLAST analysis which uses higher stringency for alignment failed to detect this similarity.

DISCUSSION

We demonstrate here a crossreactivity of antibodies against epitopes in both 4.1R and

beta-tubulin. These data indicate a similarity of the 4.1R carboxy terminus with a short region in beta-tubulin, which is further supported by the sequence alignment of the 4.1R C-terminal peptide with the helix 5 region of beta-tubulin. Our experiments with nocodazole show that after depolymerization of microtubules, the immunofluorescence staining with the 4.1R antibody is restricted to the centrosome. This suggests that the antibody can see both tubulin as well as protein 4.1R in the cell, but under normal conditions, the centrosomal 4.1R signal is obscured by the high abundance of microtubule filaments in the cytoplasm. Plasma membrane staining was not detected with this antibody, which might indicate that the extreme carboxy-terminus of 4.1R is masked in the plasma membrane skeleton.

What is the biological significance of the similarities between the 4.1R carboxy terminus and beta-tubulin? The partial localization of 4.1R to the centrosome may suggest a role in microtubule organization. Microtubules are nucleated from the pericentriolar material, but many of them are subsequently released into the cytoplasm (Keating *et al.*, 1997), potentially by the action of a microtubule severing factor, katanin (Hartman *et al.*, 1998). It is not clear how the anchoring and release of microtubules at the centrosome is regulated in detail. One possibility would be that a protein such as 4.1R is involved in microtubule release. With its carboxy terminal end showing similarity to beta-tubulin, 4.1R could mimic microtubule ends and

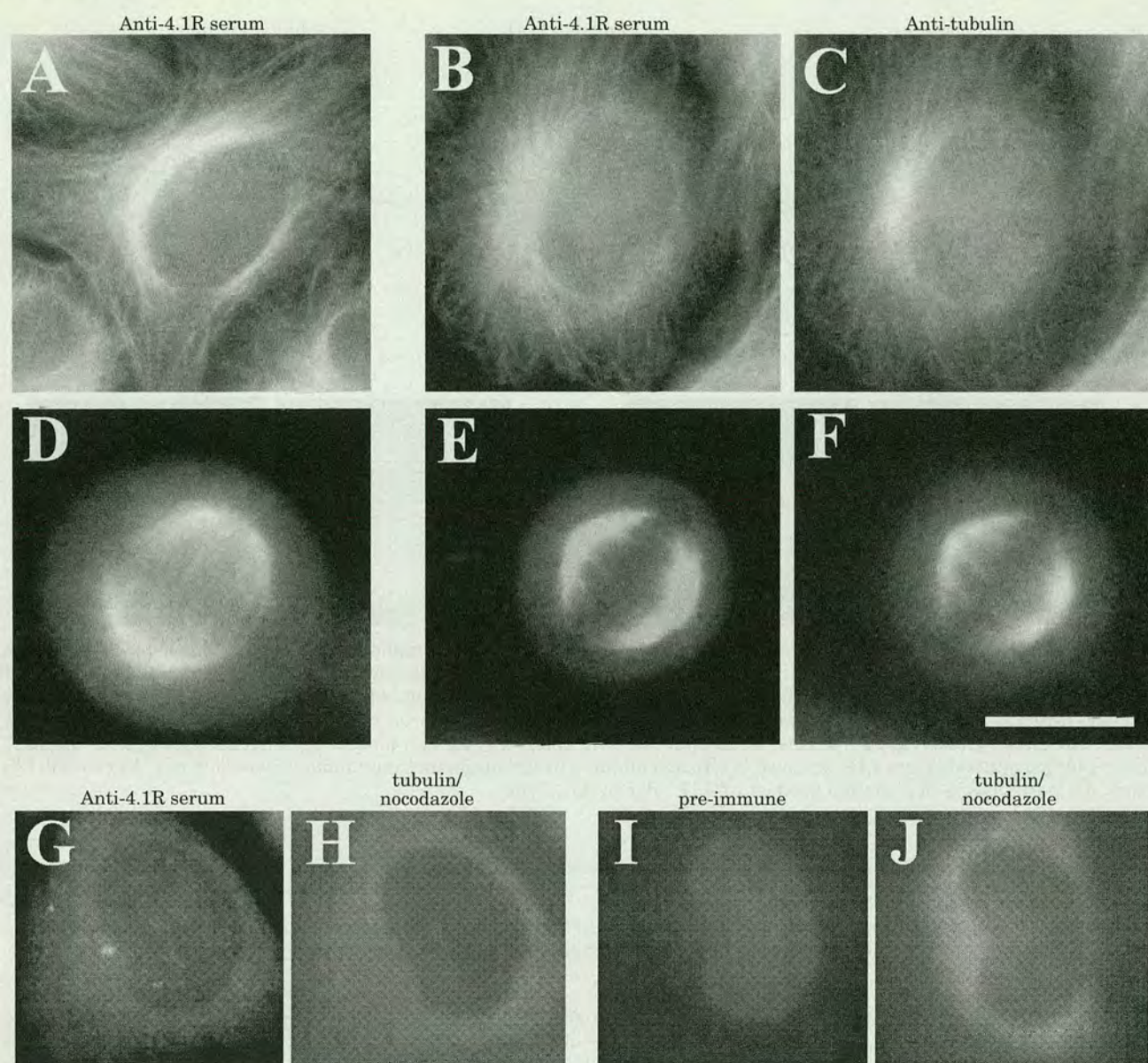


Fig. 3. Peptide antibodies raised against 4.1R stain microtubules. (A) and (D) show single immunofluorescence of the 4.1R peptide antiserum in HeLa cells during interphase (A) and mitosis (D). Double immunofluorescence with the same serum and monoclonal anti-tubulin antibody is shown during interphase in (B, C) and during mitosis in (E, F). (G) to (J) show HeLa cells treated with nocodazole. (H) and (J) show anti-tubulin staining, (G) and (I) show immunofluorescence with 4.1R peptide antiserum, or with pre-immune serum from the same rabbit, respectively. Bar in (F), 20 μ m; same magnification in (A)–(J).

compete for the binding to centrosomal components, thus disconnecting microtubules from the centrosomal surface.

This idea is supported by recent findings from Perez-Ferreiro *et al.* (2001). These authors report that overexpression of 4.1R disturbed microtubule organization, and that microtubules no longer radiated from a single pericentriolar focus, although centrosomal markers such as pericentrin or gamma-tubulin remained unaltered. Moreover,

the carboxy-terminal region of 4.1R had the maximum capacity of interfering with microtubule organization in their experiments. Given the multitude of 4.1 isoforms and their varying subcellular localization in different cell types (Takakuwa, 2000), the 4.1 protein could contribute to the modulation of the microtubule network by releasing microtubules from nucleation sites, and therefore contribute to the various cell shapes and properties of many differentiated cells.

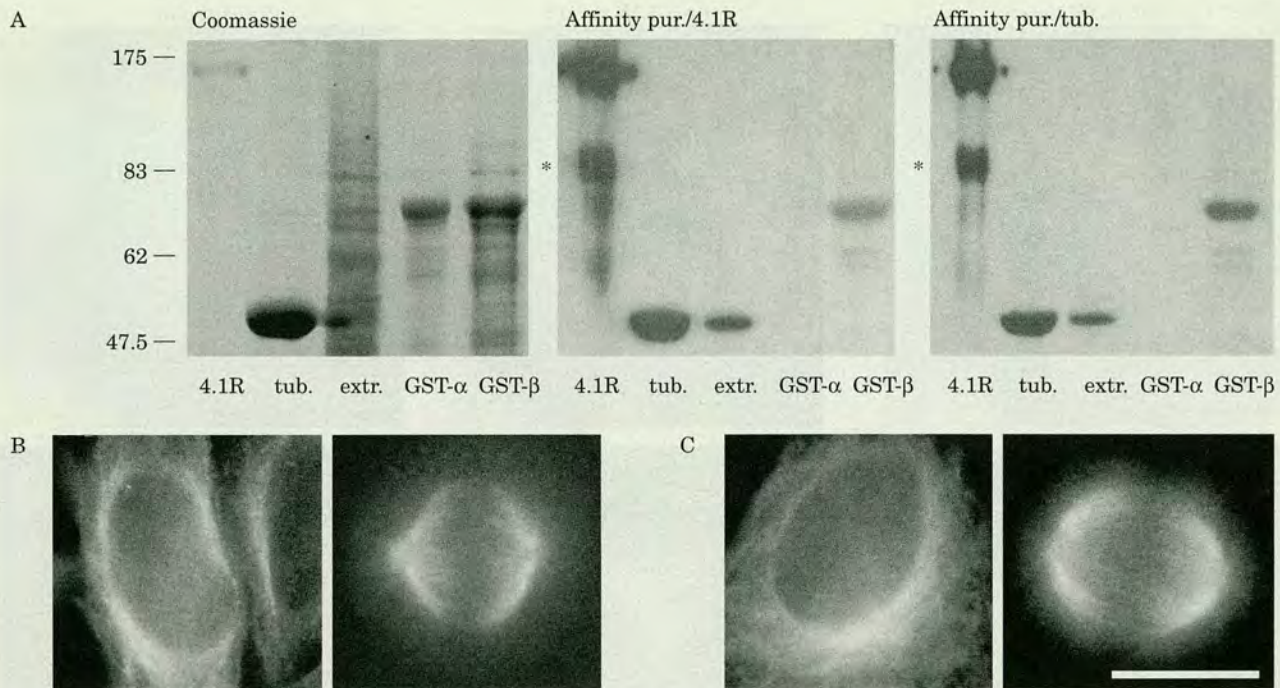


Fig. 4. Anti-4.1R antibodies cross-react with beta-tubulin. (A) shows Coomassie staining of a protein gel loaded with full-length 4.1R protein (4.1R), brain tubulin (tub.), *Xenopus* egg extract (extr.), and GST-tagged recombinant alpha-tubulin (GST- α) or beta-tubulin (GST- β), respectively. Note the increased molecular weight of the recombinant forms of tubulin after addition of the GST-tag. Identical gels were blotted and probed with 4.1R peptide antibodies, previously affinity purified from recombinant 4.1R (middle), or brain tubulin (right). The same affinity purified antibodies were used for immunofluorescence staining of HeLa cells; affinity purified antibody from 4.1R is shown in (B), and affinity purified antibody from tubulin is shown in (C). Asterisks in (A) denote the position of a degradation product of 4.1R. Bar in (C), 20 μ m.

		helix 5	sheet 6	helix 6	helix 7	
α -tubulin	177	VSTAVVEPYNSILTT HTTLE HSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLISQIVS				236
β -tubulin	175	VSDTVVEPYNATLS VHQ LVENTDETYCIDNEALYDICFRTLKLTTPTYGDLNHLVSATMS				234
4.1R peptide		SVTKGV VHQ ETEIA				

Fig. 5. Alignment of the C-terminal 4.1R peptide with amino acids 177 to 236 of alpha-tubulin (identical in mouse, humans, chicken, and *Xenopus*), and the corresponding region in beta-tubulin (amino acids 175 to 234). The positions of alpha-helices and beta-sheets in tubulin are indicated. Identical amino acids are highlighted in bold typescript.

The binding of 4.1R to NuMA, as reported by Mattagajasingh *et al.* (1999), could provide an additional regulatory element to control microtubule behaviour. Whereas only small amounts of a NuMA splice variant are found at the centrosome during interphase (Tang *et al.*, 1994), the majority of NuMA localizes near the centrosome in mitosis. In contrast to NuMA, the protein 4.1R appears to be a constitutive resident of the centrosome throughout the cell cycle, binding to the protein CPAP (Hung, 2000). Therefore, the protein 4.1R could provide a docking site on the mitotic centrosome for NuMA protein complexes that are transported towards the spindle poles by dynein/dynactin (Merdes *et al.*, 2000). Similar to

interphase cells, the NuMA-4.1R interaction in mitosis might affect processes such as microtubule release from the centrosome (Mastronarde *et al.*, 1993), or poleward flux of tubulin polymer, as proposed by Sawin *et al.* (1992).

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